AN ABSTRACT OF THE THESIS OF

Michael P. LeMaster for the degree of Doctor of Philosophy in Zoology presented on September 21, 2001. Title: Phenotypic Variation in the Sexual Attractiveness Pheromone of the Red-sided Garter Snake, *Thamnophis sirtalis parietalis*.

Abstract approved: Redacted for Privacy

Robert T. Mason

Pheromones are chemical cues produced by organisms that affect the behavior and/or physiology of conspecifics. The orchestration of reproductive behaviors in many animals depends on the expression of sex pheromones. In insects, intraspecific variation in sex pheromone expression is commonly observed and often influences social interactions between individuals. To what extent similar variation is present in vertebrate sex pheromone systems is not well understood. This thesis investigated the occurrence and significance of phenotypic variation in the sexual attractiveness pheromone of the red-sided garter snake, *Thamnophis sirtalis parietalis*. Composed of a homologous series of saturated and unsaturated methyl ketones sequestered in the skin lipids of females, this pheromone elicits male courtship behaviors during the breeding season.

Variation in the expression of the sexual attractiveness pheromone was examined at three levels: seasonal, individual and populational. Seasonal variation in pheromone expression was examined with respect to trailing behavior. In field experiments, males followed female trails during the breeding season but not during the non-breeding season. Skin lipid analysis revealed marked differences in pheromone composition between the

two seasons, suggesting a role for this pheromone in regulating male trailing behavior. Individual variation in pheromone expression was examined with respect to mate choice. In arena trials, males displayed a courtship preference for larger females and continued to demonstrate this preference when visual, tactile, and behavioral cues from the females were removed through the use of skin lipid extracts. Analysis of pheromone profiles from individual females showed size-specific variation in composition, demonstrating that this pheromone can function as a reliable indicator of female size. Finally, populational variation in pheromone expression was examined with respect to sexual isolation among hibernacula. In arena and trailing experiments, males preferred to court and trail resident (same den) females over non-resident (distant den) females. Chemical analysis showed significant inter-den variation in pheromone composition, suggesting that this pheromone represents the cue that males use to identify females from their own population. In summary, these studies demonstrate that significant variation exists in the female sexual attractiveness pheromone of the red-sided garter snake and suggest that this variation has functional significance for this species.

Phenotypic Variation in the Sexual Attractiveness Pheromone of the Red-sided Garter Snake, *Thamnophis sirtalis parietalis*

by

Michael P. LeMaster

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

Presented September 21, 2001
Commencement June 2002

Doctor of Philosophy thesis of Michael P. LeMaster presented on September 21, 2001.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Michael P. LeMaster, Author

Acknowledgements

I would first like to thank my doctoral advisor, Robert T. Mason. His encouragement and support has been the driving force behind my development as an academic during my tenure at Oregon State University. In addition, I would also like to thank my many committee members – Stevan J. Arnold, Lynne D. Houck, Michael S. Blouin, Martin S. Fitzpatrick, David E. Williams, and Peter B. McEvoy – for the contributions they have made toward my dissertation.

I also thank Michael S. Blouin and his laboratory (especially Eric Hoffman, Kirsten Monsen, and Wolfe Wagman) for allowing a chemical ecologist to invade their molecular world and run amok for two years. Kudos also to William H. Gerwick for allowing me access to his gas chromatograph/mass spectrometer, Richard Shine and his laboratory for intellectual and physical assistance during our many springs cavorting with the garter snakes up north, and the entire Chatfield, Manitoba crew (especially Al and Gerry Johnson and Dave Roberts) for providing a wonderful atmosphere in which to conduct our research.

I will forever be grateful to my fellow graduate students in the Mason laboratory, with whom I spent many an enjoyable day working hard or, more often than not, hardly working – Mike Greene, Ignacio Moore, Darren Lerner, Heather Waye, Deborah Luttershmidt, and Ryan O'Donnell. I am also indebted to the undergraduates and research associates in the laboratory who have contributed both intellectually and physically to my research – Brian Carter, Jennifer Penn, Amy Price, Hope Rieden, Shantel Stark, and Kevin Sutherland.

Sigma Xi (Grants-in-Aid of Research) and the OSU Zoology Research Funds provided part of the funding for this thesis – to them, a big thanks for seeing value in my research. I also thank Rick Jones for masterfully drawing the figures presented in chapters 3, 6 and 7 of this thesis. In addition, a heartfelt thanks to the zoology office staff, particularly Diane Rowe and Tara Bevandich, for assisting me in my research/teaching needs and extinguishing the administrative fires I had a knack for lighting on a weekly basis.

I would also like to acknowledge the friendship of Tom and Deborah Roberts, Adam Jones, Ginger Carney, Alan Verde, DeLaine Larsen, Lisa Belden, Barb Taylor, and Randy Bender. With them I spent many an hour riding and running through the mountains, working out at the "Dixon Library", visiting the local eateries (*e.g.,* Tarn Tip) and watering holes (*e.g.,* Bombs Away), being true Beaver Believers (Go Beavs!), and just kicking back in Cordley Hall. Without them, I would not have maintained the semblance of sanity that I have left.

Lastly, but most importantly, I extend a heartfelt thanks to my wife, Julie LeMaster, and my immediate (Bill and Margie LeMaster; Marcus LeMaster and Diane Davidson) and extended (Don and Mary Jane Isensee; Pete, Kristi, Alisha, and Tayla Isensee; Mike Isensee and Jana Schofield) families for their love, support, encouragement, and continued patience with me throughout this entire process.

Contribution of Authors

Ignacio T. Moore contributed to the design and data collection of chapter two. Robert T. Mason has served as my graduate advisor in the Department of Zoology at Oregon State University.

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Dedication

This thesis is dedicated to my parents Bill and Margie LeMaster and my wife Julie LeMaster in acknowledgement of their love, support, and encouragement throughout the years

Phenotypic Variation in the Sexual Attractiveness Pheromone of the Red-sided Garter Snake, *Thamnophis sirtalis parietalis*

Chapter 1 – Introduction

General Background

The behaviors of animals are strongly influenced by stimuli from the external environment. Animals gather this information with their sensory systems. In the past, considerable attention has been focused on the effects of visual and auditory cues on animal behavior (*e.g.*, Thomas *et al.*, 1992; Ziegler & Bischof, 1993; Kroodsma & Miller, 1996; Ryan, 2001). However, limited progress has been made on the subject of chemical communication, especially with reference to vertebrates. It is becoming increasingly clear that chemical communication plays an important role in vertebrates (Albone, 1984), and in some species it may be the dominant mode of communication between individuals (*e.g.*, reptiles – Mason, 1992).

One group of chemical cues often utilized by vertebrates, particularly with respect to reproduction, is pheromones. Pheromones are chemical cues, or semiochemicals, produced by one individual that affect the physiology and/or behavior of a conspecific (Karlson & Lüscher, 1959). Pheromones may function either as primers, eliciting a change in a recipient's physiological state over time (*e.g.*, milt production in fish – DeFraipont & Sorensen, 1993), or releasers, eliciting an immediate behavioral response from a recipient (*e.g.*, clasping behavior in newts – Thompson *et al.*, 1999). Depending on its biochemistry, a pheromone can volatilize in air or diffuse through water for long-distance communication (olfactory pheromone – Mustaparta, 1984), or can linger on the

sender, requiring the receiver to come in contact with the sender before the signal can be detected (contact pheromone – Stadler, 1984).

To date, the vast majority of research concerning pheromones has been conducted using insects as model systems (Bell & Cardé, 1984; Roitberg & Isman, 1992; Eisner & Meinwald, 1995). The first pheromone to be isolated and identified was a sex pheromone for the silk moth, *Bombyx mori* (Butenandt *et al.*, 1959). Since that time, well over one thousand insect pheromones have been identified and characterized (Abelson, 1985). Indeed, sex attractiveness pheromones alone have been identified in over 1600 insect species from 90 families in nine orders (Mayer & McLaughlin, 1991). Knowledge of the chemical structure of pheromones has allowed investigators to make significant advances in understanding how these chemical cues regulate insect behavior (Bell & Cardé 1984; Roitberg & Isman, 1992).

In contrast to the abundance of pheromones identified in insects, very few vertebrate pheromones have been isolated and identified. These include pheromones for fish (*e.g.*, goldfish – Stacey & Sorenson, 1986), amphibians (*e.g.*, newts – Kikuyama *et al.*, 1995; salamanders – Rollmann *et al.*, 1999; frogs – Wabnitz *et al.*, 1999), reptiles (*e.g.*, snakes – Mason *et al.*, 1989) and mammals (*e.g.*, pig – Patterson, 1968; elephant – Rassmussen *et al.*, 1996). The dearth of identified vertebrate pheromones is primarily due to the multicomponent sensory nature of vertebrates in which a combination of sensory inputs (*e.g.*, visual, tactile, chemical) are often responsible for mediating a particular behavior (Albone, 1984). Such diversification makes it difficult to establish effective bioassays for measuring behavioral responses of individuals to isolated chemical cues in order to determine how such cues are utilized.

The lack of basic structural knowledge of vertebrate pheromones has hampered our efforts to understand vertebrate pheromone systems and has left many basic areas of research unexplored. Few studies have attempted to address intraspecific variation in the chemical composition of a vertebrate pheromone and the potential significance of such variation. Intraspecific variation in other modes of sensory input has been studied extensively in vertebrates, leading to the discovery of multiple roles for the variation. For example, females of many bird species utilize variation in visual traits to assist in selecting among potential suitors (*e.g.,* color intensity – Hill, 1990; tail length – Møller, 1988) and many frogs utilize variation in auditory traits for mate selection (*e.g.,* call rate – Sullivan, 1983; call pitch – Ryan, 1980). It is reasonable to conclude from these and other studies that similar functional variation may also be present in vertebrate chemical communication systems.

Intraspecific Variation in Pheromone Expression

Intraspecific variation in pheromone production and expression has been examined at length in insects, particularly with reference to sex pheromones. Not surprisingly, studies examining large-scale temporal variation in sex pheromone expression show a close relationship between pheromone production and gonadal cycles (*e.g.,* Blomquist *et al.*, 1987; Schal *et al.*, 1991), indicating that pheromone production is limited to the breeding periods. In addition, small-scale temporal variation has been observed for individuals during a single breeding period. These studies have showed that as the breeding season progresses, individuals exhibit variation in the quantity (*e.g.,* Webster & Cardé, 1982; Schal *et al.*, 1987; Kamimura & Tatsuki, 1993) and/or quality

(Kuwahara, 1979; Jackson & Bartelt, 1986) of the pheromone they are emitting. Often such variation is used by receivers to discriminate among signalers at various stages in their reproductive period (*e.g.*, Gemeno & Haynes, 2000).

Individual variation has also been examined in insect sex pheromones by sampling individuals at the same point in their reproductive cycle to control for temporal variation. Although such studies have examined variation in the quantity of pheromone produced and emitted by individuals (*e.g.*, Barrer *et al.*, 1987; Ono *et al.*, 1990; Svensson *et al.*, 1997), the majority of studies examining individual variation have focused on component blend differences. For some species, a relatively precise blend of compounds has been observed among individuals (*e.g.*, Miller & Roelofs, 1980; Linn *et al.*, 1986), suggesting that such precision is required for mate-finding in these species. Alternatively, other species appear to utilize a more broadly-tuned signal, with senders producing, and receivers responding to, an extensive range of pheromone blends (*e.g.*, Löfstedt *et al.*, 1985; Attygale *et al.*, 1986; Guerin *et al.*, 1986). Even with a broadly-tuned signal, receivers in these systems are often able to discriminate among particular blends, allowing for receivers to discriminate among senders based on signal quality (*e.g.*, mate choice – Löfstedt *et al.*, 1989; Moore & Moore, 1999).

The greatest volume of work examining intraspecific variation in emitted pheromone blends within insect sex pheromone systems has occurred at the population level. Such studies have often observed that pheromone emissions remain fairly constant throughout an insect's geographical range (*e.g.*, Roelofs & Cardé, 1974; Miller & Roelofs, 1980; Haynes & Hunt, 1990). This consistency may be favored by gene flow between populations and/or mutually imposed stabilizing selection between signaler and

responder (Haynes & Hunt, 1990). In contrast, the sex pheromone emissions in other insect species display geographic variation (Lanier *et al.*, 1972; Löfstedt *et al.*, 1986; Huang *et al.*, 1998), possibly resulting from directional selection and/or genetic drift acting on isolated populations (Haynes & Hunt, 1990). The biological significance of populational variation in sex pheromone expression among species varies. In some instances, the observed variation does not appear to affect interpopulational reproductive interactions (*e.g.*, Gemeno *et al.*, 2000). In other instances, however, courtship disruption has been observed between individuals from different geographical locations, leading to various degrees of sexual isolation between the populations (*e.g.*, incomplete sexual isolation – Tóth *et al.*, 1992; Wu *et al.*, 1999: complete sexual isolation – Frérot & Foster, 1991).

To date, only a single study has examined intraspecific variation in a vertebrate pheromone. Rollmann *et al.* (2000) examined interspecific and populational variation in a courtship pheromone, the plethodontid receptivity factor (PRF), in Plethodon salamanders of the *jordani-glutinosus* complex. Composed of multiple isoforms of a 22 kD protein (Rollmann *et al.*, 1999), this pheromone is responsible for increasing female receptivity during the breeding season (Houck *et al.*, 1998). This pheromone was found to vary considerably between geographically isolated populations and species, and was remaining relatively stable within populations. It is suggested that this observed variation might play a role in the moderate level of sexual isolation previously observed among populations of this complex (Rollmann *et al.*, 1999). However, behavioral trials testing the biological significance of these pheromonal differences have yet to be performed.

The Sexual Attractiveness Pheromone of the Red-sided Garter Snake

Snakes are excellent models for vertebrate pheromone research because the initiation of many behaviors in snakes is dependent on pheromone production and expression (reviewed in Carpenter & Ferguson, 1977; Burghardt, 1980; Mason, 1992). In particular, the reproductive success of snakes depends on the production and perception (via the vomeronasal organ – Halpern, 1987) of specific sex pheromones. These pheromones coordinate multiple activities in the reproductive process, including locating potential mates over long distances (*e.g.,* Gehlback *et al.*, 1971; Ford, 1981), eliciting courtship behavior once mates come in contact (*e.g.,* Noble, 1937; Andrén, 1986), and terminating courtship once mating is complete (*e.g.,* Ross & Crews, 1977; Shine *et al.*, 2000).

Garter snakes (genus *Thamnophis*) are one of the most commonly encountered snakes in North America with a continuous range stretching from central Canada to Costa Rica (Rossman *et al.*, 1996). Since the 1930's, investigators have demonstrated that within this genus the initiation of male courtship behavior depends on the production and expression of female sexual attractiveness pheromones (Noble, 1937; Garstka & Crews, 1981; Mason *et al.*, 1989). When a male comes in contact with a female expressing such a pheromone, the male displays courtship behavior characterized by increased tongue-flick rate, chin-rubbing along the dorsum of the female, and alignment of cloacal openings (Noble, 1937). These behaviors are simultaneously expressed only in a reproductive context in response to the sexual attractiveness pheromone (Camazine *et al.*, 1980).

Chemical extracts from the skin of an attractive female garter snake, when presented on an artificial substrate (*e.g.,* filter paper) will elicit male courtship behavior with the same intensity as shown to the actual female (Noble, 1937; Mason & Crews, 1985). The fact that males in this genus respond with robust, stereotypical behaviors in such situations allows for the establishment of well-defined behavioral bioassays with which to test a male's response to isolated chemical cues. Such a bioassay can also facilitate the process of pheromone isolation and identification through the response-guided strategy (Albone, 1984). In this process, a chemical extract containing a pheromone is fractionated and presented in a bioassay to determine which fraction contains the active pheromone. This is repeated (fractionation/bioassay presentation) until the biologically active components are isolated, which can be subsequently identified.

Mason *et al.* (1989) successfully utilized this strategy to isolate and identify the female sexual attractiveness pheromone of the red-sided garter snake (*Thamnophis sirtalis parietalis*). To date, this represents the only pheromone to be characterized in a reptilian species. Composed of a homologous series of long-chain saturated and ω-9 cis-unsaturated methyl ketones, the sexual attractiveness pheromone is sequestered in the skin lipids of the female during the breeding season (Mason *et al.*, 1989; Mason *et al.*, 1990). Although both saturated and unsaturated methyl ketones are required to elicit full male courtship behavior from male red-sided garter snakes, unsaturated methyl ketones appear to be the more biologically active of the two groups. When presented in isolation, unsaturated methyl ketones elicit a five-fold increase in male response over saturated methyl ketones (Mason *et al.*, 1989).

In the series of studies presented for this thesis, I investigated the occurrence and potential significance of intraspecific pheromonal variation in the sexual attractiveness pheromone of the red-sided garter snake. This species is the most northern-living reptile in the Western Hemisphere with a range extending into central Manitoba, Canada (Logier & Toner, 1961). The annual aggregations of red-sided garter snakes at underground hibernacula in Manitoba are unique natural phenomena representing the highest concentration (up to 20,000 individuals per den) of snakes in the world (Gregory, 1984). Marshes, shallow lakes, and areas with poor drainage offer good summer feeding grounds for the snakes, while limestone bedrock provides hibernation sites where the snakes are constrained to spend up to eight months of the year to avoid the harsh winters (Aleksiuk & Stewart, 1971).

Summary of Research and Experimental Goals

The unifying goal of this thesis was to determine to what extent intraspecific variation exists in the female sexual attractiveness pheromone of the red-sided garter snake and to evaluate the potential significance of this variation. To this end, the occurrence and potential significance of variation in the sexual attractiveness pheromone was examined at three levels: 1) seasonal, with respect to male trailing behavior, 2) individual, with respect to male mate choice, and 3) populational, with respect to sexual isolation among hibernacula.

Chapter two of this thesis explores conspecific trailing behavior of the red-sided garter snake on its natural substrate. Previous laboratory work has demonstrated that male garter snakes are able to detect and respond to female trails during the breeding

season (*e.g.*, Noble, 1937; Ford, 1981, 1982) and, to an extent, during the non-breeding season (*e.g.*, Constanzo, 1989; but see Halpern, 1992). However, the trailing behavior of male snakes during these two seasons has yet to be tested under natural conditions. Thus, my aim in this study was to ascertain whether conspecific trailing behavior is biologically significant to male red-sided garter snakes in their natural environment during the breeding and non-breeding seasons.

Chapter three investigates the role of the female sexual attractiveness pheromone in mediating male trailing behavior. Similarities in the location of pheromone expression (dorsal surface), season of activity (breeding season), and sexual dimorphism in pheromone production (female) and detection (male) have led a number of investigators to hypothesize that a single pheromone elicits both trailing and courtship behavior in this species (Noble, 1937; Garstka & Crews, 1981; Mason, 1992). To test this hypothesis, I investigated the trailing response of male red-sided garter snakes to 'she-males'. She-males are a subset of males morphologically and anatomically indistinguishable from other males, but they express the sexual attractiveness pheromone on their dorsal surface during the breeding season (Mason, 1993). If the sexual attractiveness pheromone is the pheromone responsible for mediating trailing behavior, then I hypothesized that males should follow 'she-male' trails to the same extent as female trails.

Chapter four examines to what extent seasonal variation exists in the composition of the female sexual attractiveness pheromone of the red-sided garter snake. If males do utilize this pheromone to trail females during both the breeding and non-breeding seasons, then I would expect to see very similar pheromone profiles across the two seasons. Alternatively, if males only utilize this pheromone to locate females during the

breeding season, then I might expect to observe significant variation in the composition of this pheromone between the two seasons.

Chapter five evaluates the role of the female sexual attractiveness pheromone in mediating male mate choice in the red-sided garter snake. During the breeding season, males of this species do not randomly associate with females, but instead display a courtship preference for larger females (Hawley & Aleksiuk, 1976; Shine *et al.*, 2001). The dependence of males on the sexual attractiveness pheromone to elicit courtship behavior suggests that this is the cue utilized by males to differentiate among potential mates. Utilizing arena trials, I tested the courtship response of males to skin lipid extracts from large and small females to determine whether males are able to distinguish among females of varying size based on chemical cues alone. Pheromone samples were also collected from individual females and analyzed to determine whether the sexual attractiveness pheromone contains the necessary variation to function as a reliable indicator of female body size.

Finally, Chapter six examines whether pheromonally-mediated sexual isolation exists between denning populations of red-sided garter snakes in Manitoba. The reliance of this species on the sexual attractiveness pheromone to initiate reproductive behavior, coupled with its discontinuous population structure in Manitoba, offers a strong basis for testing this hypothesis. Utilizing arena trials, I first investigated whether male red-sided garter snakes from two geographically isolated hibernacula displayed a courtship preference for females from their own den versus females from the distant den. Because a courtship preference was detected, I then set out to determine whether the observed preference is mediated through variation in the sexual attractiveness pheromone. This

was accomplished by 1) utilizing trailing experiments to determine whether the observed preference is mediated through chemical cues, and 2) performing chemical analysis to evaluate whether detectable variation exists in the female sexual attractiveness pheromone between the two hibernacula.

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Chapter 2

Conspecific Trailing Behavior of Red-sided Garter Snakes (*Thamnophis sirtalis parietalis*) in the Natural Environment

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Published in *Animal Behaviour*,
Plenum Press, New York, New York
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2001 – Volume 61: 827-833

Abstract

The ability of animals to orient based on pheromonal cues in the environment can have significant consequences towards their reproductive success and survival. For example, it is hypothesized that snakes utilize pheromone trails to locate potential mates during the breeding season and to locate winter hibernacula. Numerous studies have demonstrated that snakes are capable of detecting and following pheromone trails in the laboratory, but it has yet to be shown experimentally whether snakes utilize such behavior in their natural environment. In this study, we conducted trailing experiments to test the response of adult red-sided garter snakes (*Thamnophis sirtalis parietalis*) to pheromone trails on the natural substrate under natural conditions during two periods, the spring breeding season and the autumn migration back to the hibernacula. Male snakes detected and followed female trails in the spring, while neither sex displayed trailing behavior during the period of autumn migration. Our results suggest that adult garter snakes utilize pheromone trails for locating potential mates during the breeding season and most likely rely on other mechanisms (*e.g.*, visual cues) for navigation to winter hibernacula.

Introduction

An animal's survival and reproductive success is strongly dependent on its ability to locate necessary resources. Many vertebrates use chemical signals to orient towards potential resources, which may include mates (*e.g.*, frogs – Wabnitz *et al.*, 1999), food (*e.g.*, rattlesnakes – Chiszar *et al.*, 1990), breeding sites (*e.g.*, salmon – Hasler, 1960) and refuge (*e.g.*, pigeons – Papi, 1982). One type of chemical signal often utilized by

vertebrates for orientation is pheromones. Pheromones are semiochemicals released by individuals that affect the physiology and/or behavior of members of the same species (Karlson & Luscher, 1959). Once deposited in the environment, a pheromone trail can guide a receiver directly to the location of the conspecific producer.

Conspecific trailing behavior is widespread in snakes, with numerous species within at least five families demonstrating the behavior (Ford, 1986). In snakes, conspecific trailing behavior is mediated by pheromones sequestered on the skin, which are laid down on the substrate along with skin lipids as a snake passes across the substrate (Noble, 1937). Subsequent snakes encountering the trail are able to transport the pheromonal cues via tongue flicking to the vomeronasal organ in the roof of the mouth where the cues are detected (Halpern & Kubie, 1983). Snakes are believed to utilize pheromone trails for aggregating, locating potential mates during the breeding season and locating winter hibernacula (reviewed in Ford, 1986; Mason, 1992).

Most reports document the occurrence of conspecific trailing behavior during the breeding season and describe male snakes seeking out sexually attractive females (*e.g.*, Noble, 1937; Ford, 1981, 1982; Lillywhite, 1985; Andrén, 1982, 1986). Less is known about the role of pheromone trails in locating winter hibernacula. Communal denning is a strategy often utilized by northern temperate snakes to escape harsh winter conditions experienced at higher latitudes (Gregory, 1984), and conspecific trailing behavior has long been suspected to aid snakes during travels to the hibernacula (Noble & Clausen, 1936; Hirth, 1966). Previous laboratory work has demonstrated that both juvenile snakes (Heller & Halpern, 1981; Brown & MacLean, 1983; Graves *et al.*, 1986) and adult snakes

(Ford, 1981; Constanzo, 1989) are able to detect and respond to pheromone trails during periods when snakes are actively migrating.

Here, we report two experiments designed to investigate conspecific trailing behavior in the natural environment for the red-sided garter snake, *Thamnophis sirtalis parietalis*. To our knowledge, this is the first study to field-validate laboratory and anecdotal observations of conspecific trailing behavior in snakes. The need to field-validate laboratory observations in behavioral ecology has received recent attention (*e.g.*, fish alarm pheromones – Magurran *et al.*, 1996; Smith, 1997), raising questions concerning the role of experimentation in behavioral ecology studies (Ylönen & Wolff, 1999). With this study, we are attempting to ascertain whether conspecific trailing behavior is biologically significant to red-sided garter snakes in their natural environment.

Our two major aims in this study are to 1) determine whether red-sided garter snakes respond to pheromone trails in the natural environment and 2) evaluate the role of conspecific trailing behavior for this species in locating potential mates during the breeding season and locating over-wintering hibernacula. Annual aggregations of red-sided garter snakes at underground hibernacula in Manitoba, Canada are unique natural phenomena representing the highest concentration of snakes in the world (Gregory, 1984). For these populations, mating occurs at the hibernacula immediately following spring emergence (Gregory, 1977). After mating, the snakes disperse to the summer ranges which can be quite distant from the hibernaculum (>15 kilometers – Gregory & Stewart, 1975), before returning in early autumn along well defined travel lanes to the same hibernaculum utilized over previous winters (Gregory, 1974; Macmillan, 1995). If

pheromone trails are utilized in the natural environment for locating mates and winter hibernacula, then red-sided garter snakes should detect and follow pheromone trails presented on the natural substrate under naturally occurring conditions during the spring breeding season and the period of autumn migration back to over-wintering hibernacula.

Materials and Methods

Study Population and Site

We conducted our research at the Narcisse Wildlife Management Area, in the Interlake region of Manitoba, Canada (50°44'N, 97° 34'W). The Narcisse Wildlife Management Area contains three hibernacula in close proximity to one another, with each hibernaculum possessing in excess of twenty thousand red-sided garter snakes during the winter months (R.T. Mason, unpublished data).

All trailing experiments were performed on the natural substrate (tall grass prairie – Shay, 1984) adjacent to the hibernacula in May (breeding season) and September (period of migration) of 1997. Testing days and times of day (1000 hrs to 1600 hrs) were chosen so that all trials were conducted under similar ambient conditions (mostly sunny skies with light winds and temperatures of 15-20°C) which correspond with conditions when red-sided garter snakes are most active. We collected adult snakes (snout-vent length (SVL) > 40.0 cm for males; SVL > 50.0 cm for females) on the morning that behavioral tests were to be performed and returned them to the place of collection following testing. Spring individuals (n = 72) were collected at the hibernacula as the snakes emerged from winter dormancy and autumn individuals (n = 92) were collected

approximately 800 meters from the hibernacula as the snakes actively migrated back to the hibernacula.

Behavioral Trials

In both the spring and autumn, we tested the response of 1) males to female trails, 2) males to male trails, 3) females to female trails and 4) females to male trails. Snakes were tested on plots mapped out on the natural substrate using small flags. Each plot consisted of a semi-circle with a radius of three meters (Figure 2.1). A small, opaque box with a single opening onto the semi-circle was placed at the radial center of the semi-circle. All plots were laid out facing a northeasterly direction with each trial performed on an independent plot. Plots were faced in the same direction for the sake of uniformity after initial examinations showed no difference in the behavior of the snakes across multiple compass headings.

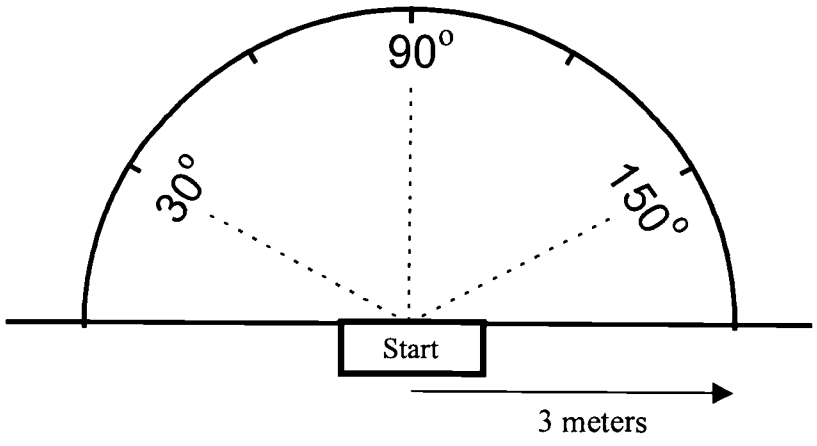

Figure 2.1. Diagram of experimental plot used for trailing experiments. Plots were established in a natural field with snake trails laid down randomly in one of three directions (30 degrees, 90 degrees, or 150 degrees).

For each trial, we randomly laid down a snake trail at one of three angles: 30 degrees, 90 degrees, or 150 degrees. We chose multiple angles to control for external factors (*e.g.*, solar cues, landmarks) that might bias the direction of the test snake. Trails were laid down by randomly selecting a stimulus snake (male or female depending on the treatment) and sweeping it through the grass once, beginning at the exit to the hide box and ending at the edge of the semi-circle at the appropriate angle. Care was taken to cover the cloacal opening of the snake so that contamination from cloacal gland secretions did not occur. A second 'trail' was also laid down in each plot by sweeping our index finger through the grass in the same manner that the real stimulus was applied, but at a different angle from the stimulus trail. This was done to control for mechanical disturbance to the substrate and human skin lipid contamination.

Once the trail was established, we placed a randomly chosen test snake into the opaque box and allowed it to acclimate for five minutes. The opening onto the semi-circle was then uncovered and the snake was allowed to exit of its own accord. Trials ended when the test snake exited the arc of the semi-circle. A unique test snake was used for each trial. Trials in which the test snake failed to exit the hide box after ten minutes or trials in which the test snake exited out the back of the semi-circle were eliminated (< 6% of trials completed). The exit location on the arc of the semi-circle was marked and the angle of exit was recorded to the nearest five degrees for each trial. We also noted whether the test snake displayed a trail contact response (TCR), an overt response characterized by careful investigation of the substrate by the snake with short, rapid tongue-flicks followed by subsequent investigative movement along the pre-existing trail (Brown & MacLean, 1983). Trail contact responses are commonly observed in snakes

actively trailing and are used by some investigators to define when an individual is trailing (*e.g.*, Lillywhite, 1985).

Fifteen trials were performed for each treatment in the spring experiment with five trials conducted at each of the three angles. Ten to thirteen trials were performed for each treatment in the autumn experiment with the trials spread systematically over the three angles.

Statistics

Relationships between the stimulus trail angle and the angle of exit for the test snakes were examined through Chi-squared tests of independence utilizing contingency tables (3x3 tables; columns = stimulus trail angles (30°, 90°, 150°) and rows = exit angle of test snakes (0-60°; 61-120°; 121-180°)) (Zar, 1984). Level of significance was set at $P < 0.05$.

Results

Spring Treatments

During the breeding season, males responded significantly to female trails, exiting within 5 degrees of the female trail in 14 of the 15 trials performed ($\chi_4^2 = 25.000$, $P < 0.001$; Figure 2.2a). All 15 males displayed the trail contact responses (TCR) immediately upon first exiting the box (Table 2.1) including the one male not exiting near the female trail. Males did not, however, show a preference for male trails ($\chi_4^2 = 2.250$,

Table 2.1. Occurrence of trail contact response (TCR) by red-sided garter snakes, *Thamnophis sirtalis parietalis*, during spring and autumn trailing experiments.

Condition	Observations of Trail Contact Response	
	Spring	Autumn
Males with female trails	15 (n = 15)	0 (n = 13)
Males with male trails	0 (n = 15)	0 (n = 13)
Females with male trails	0 (n = 15)	0 (n = 10)
Females with female trails	0 (n = 15)	0 (n = 10)

P = 0.690; Figure 2.2b) and did not exhibit TCRs. The same was true for females which appeared to exit randomly in all trials and showed no TCRs when presented with male trails ($\chi_4^2 = 6.800$, P = 0.147; Figure 2.2c) or female trails ($\chi_4^2 = 4.267$, P = 0.0.371; Figure 2.2d).

Autumn Treatments

During the autumn migration, males randomly exited the semicircle when tested with a female trail ($\chi_4^2 = 4.956$, $P = 0.292$; Figure 2.3a) and did not display TCRs at any point during the trials (Table 2.1). Similar results were observed for males tested with male trails ($\chi_4^2 = 5.316$, $P = 0.256$; Figure 2.3b) and females tested with female trails ($\chi_4^2 = 1.306$, $P = 0.860$; Figure 2.3d). Females also did not display TCRs to male trails, nor exit near the trails. However, there was a statistically significant relationship between the angle of the trail and the exit angle of the test females ($\chi_4^2 = 10.889$, $P = 0.028$; Figure 2.3c) as none of the females exited within 30 degrees of a male trail.

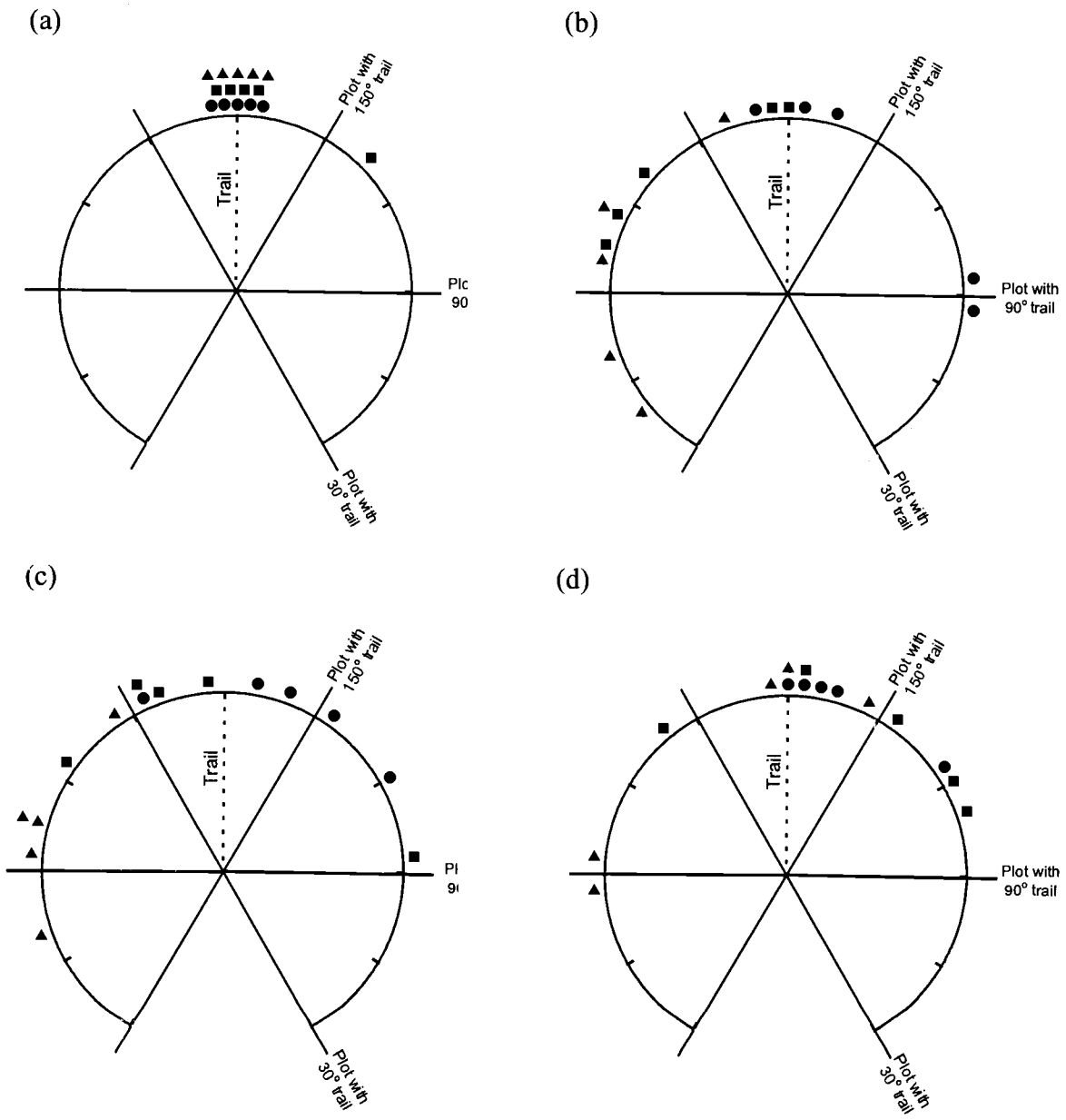

Figure 2.2. Results of spring trailing experiment showing (a) males with female trails, (b) males with male trails, (c) females with male trails and (d) females with female trails. Snakes were tested on plots with trails at either 30 degrees (●), 90 degrees (■) or 150 degrees (▲) and their exit points were marked to the nearest 5 degrees on the semi-circle. Plots have been rotated so that the trail exits are identical for all plots in a treatment.

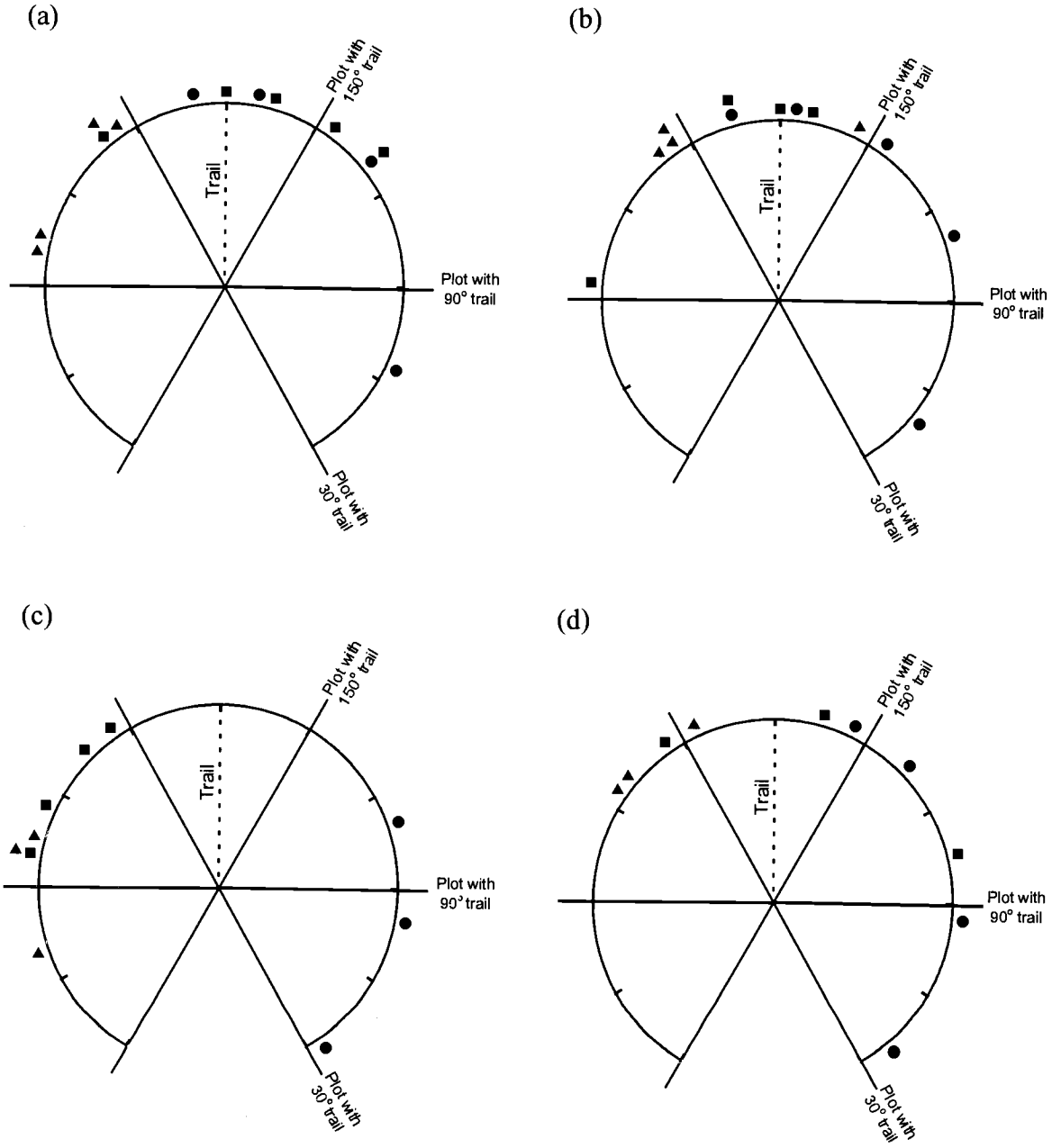

Figure 2.3. Results of autumn trailing experiment showing (a) males with female trails, (b) males with male trails, (c) females with male trails and (d) females with female trails. Snakes were tested on plots with trails at either 30 degrees (●), 90 degrees (■) or 150 degrees (▲) and their exit points were marked to the nearest 5° on the semi-circle. Plots have been rotated so that the trail exits are identical for all plots in a treatment.

Discussion

The results from this study demonstrate that adult red-sided garter snakes are able to detect and respond to conspecific trails when presented in the natural environment. We observed both sexual and seasonal variability in the response to pheromone trails. Male garter snakes displayed trail contact responses and followed trails laid down by females during the breeding season, while neither sex exhibited conspecific trailing behavior during the period of autumn migration back to the hibernacula. These results offer support for the hypothesis that adult red-sided garter snakes utilize pheromone trails for locating potential mates, but do not support the hypothesis that adult garter snakes utilize pheromone trails as the primary mechanism for locating over-wintering hibernacula.

Our results from the spring treatments confirm prior investigations of conspecific trailing in snakes during the breeding season (reviewed in Ford, 1986; Mason, 1992; Mason *et al.*, 1998). In particular, laboratory studies have demonstrated that adult male garter snakes will detect and follow female trails during the breeding season (Noble, 1937; Ford, 1978, 1981, 1982), while females in general do not trail either sex (Ford, 1986; Mason, 1992). The ability of male snakes to detect and follow female trails during the breeding season is believed to be an adaptation which allows males, limited by visual and auditory cues, to locate potential mates over long distances (Ford, 1986). This hypothesis is supported by field observations at our study hibernacula where male snakes actively following female trails will immediately initiate courtship behavior upon encountering the trail producer (M.P. LeMaster, personal observation).

The lack of response of test snakes to pheromone trails during the migratory period suggests that adult red-sided garter snakes do not rely primarily on pheromone trails for navigating to winter hibernacula. Our result contrasts with previous laboratory-based studies evaluating conspecific trailing behavior during the autumn for adult snakes (Ford, 1981; Heller & Halpern, 1981; Constanzo, 1989). Specifically, Constanzo (1989) found that adults of both sexes from a communally denning population of the common garter snake, *Thamnophis sirtalis*, followed pheromone trails when presented in the laboratory during autumn, concluding that this population of garter snakes utilizes pheromone trails for locating hibernacula.

We believe that the difference in observed behaviors between our study and previous studies arises from two fundamental differences in experimental design. First, we conducted our experiment with adult snakes that were observed to be actively migrating at the time of collection. Many garter snake species utilizing over-wintering hibernacula undergo a period of autumn mating before returning to the hibernacula (Rossman *et al.*, 1996), including the red-sided garter snake (Mendonca & Crews, 1989). It is not clear whether previous studies utilized actively migrating snakes or snakes that may still have been primarily concerned with seeking out and courting potential mates. Second, we conducted our experiment in the field, compared to previous studies conducted in the laboratory. Laboratory experiments, conducted under controlled conditions, determine whether an animal possesses the ability to respond to a particular stimulus (Smith, 1997). Field experiments, in contrast, allow for the evaluation of behavioral decisions made by an animal when influenced by prevailing environmental conditions. Thus, while garter snakes may be able to respond to pheromone trails when

presented in isolation during the autumn, additional factors co-existing in the natural environment may lessen, or eliminate, the response. In support of this hypothesis is evidence that adult red-sided garter snakes from our study population do respond to pheromone trails during the period of autumn migration when presented under laboratory conditions (M.J. Greene, personal communication).

We suggest two plausible explanations for how environmental factors might mediate the response of red-sided garter snakes to pheromone trails. First, conditions in the environment might dilute chemical cues contained within the trail to the point where they are no longer detectable (Magurran *et al.*, 1996). Alternatively, additional stimuli in the environment (*e.g.*, visual cues) may override a snake's ability to detect and process chemical cues located in trails. Most migrating vertebrates are known to rely on multiple cues for orientation, often arranged in a hierarchical structure (Able, 1991); red-sided garter snakes may preferentially seek other cues for orientation and only respond to chemical cues when the additional cues are absent. Solar cues, the most commonly used source of information for navigation by diurnally migrating animals (Able, 1980; Shöne, 1984), offer a likely source of additional information for red-sided garter snakes. In fact, studies have demonstrated that migratory species of garter snakes are capable of orienting with respect to both solar azimuth position (Lawson, 1989, 1994) and polarized light (Lawson & Secoy, 1991). A dependence on solar cues could explain why we observe red-sided garter snakes mainly migrating under sunny skies, although thermal constraints placed on snakes may also be responsible for this observation (Peterson, 1987).

While we did not observe conspecific trailing behavior during the autumn treatments, we did find a significant trend for females to exit the experimental plots well

distant of trails produced by males. Trail avoidance has previously been observed among snakes (Burger, 1989; Burger *et al.*, 1991), but these studies are limited to snakes discriminating between trails of predatory (ophiophagous) snakes and those of non-predatory (nonophiophagous) snakes. Female red-sided garter snakes may avoid male trails near the hibernacula in an effort to escape vigorous autumn courtship occasionally displayed by some males at the hibernacula (M.P. LeMaster, personal observation). It is unknown why a small subset of males continue to actively court females well after the period of autumn mating, but such harassment by these males could lead to increased mortality in females due to increased predation risk and energy expenditure (*e.g.*, Weigensberg & Fairbairn, 1994; Clutton-Brock & Langley, 1997). Alternatively, the observed avoidance may be an artifact of the experimental design. Additional studies designed to test specifically for trail avoidance behavior need to be performed to evaluate the importance of the observed result.

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Chapter 3

Evidence for a Female Sex Pheromone Mediating Male Reproductive Trailing Behavior in the Red-sided Garter Snake, *Thamnophis sirtalis parietalis*

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Published in *Chemoecology*,
Birkhäuser Publishing Ltd., Basel, Switzerland
Reprinted with permission
2001 – Volume 11: 149-152

Abstract

Male garter snakes locate females during the breeding season utilizing conspecific trailing behavior. It has been hypothesized that the female-derived chemical cue responsible for mediating male reproductive trailing behavior is the sexual attractiveness pheromone, a previously characterized contact pheromone responsible for releasing male courtship behavior. To examine this hypothesis, we tested the response of male red-sided garter snakes, *Thamnophis sirtalis parietalis*, to pheromone trails produced by males, females and 'she-males'. She-males are a small subset of males in the natural population that are morphologically and behaviorally similar to other males except that they produce and express the sexual attractiveness pheromone during the breeding season. When tested on a Y-maze, males were found to detect and follow the skin lipid trails of females and she-males, but displayed no behavioral responses to male trails. In addition, males were unable to discriminate between she-male and female trails when given a choice. These results are consistent with the hypothesis that the sexual attractiveness pheromone is the chemical cue primarily utilized by males to mediate reproductive trailing behavior.

Introduction

Snakes of the genus *Thamnophis* (garter snakes) utilize pheromone trails extensively for mediating reproductive activity (reviewed in Ford, 1986; Mason, 1992). For example, female garter snakes produce pheromone trails that guide males to their exact location during the breeding season (*e.g.*, Ford, 1981; Ford & Low, 1983; Ford & O'Bleness, 1986; LeMaster & Mason, 2001). Pheromone trails not only communicate the sex and travel direction of a trail-producing individual to male garter snakes, but also

contain information concerning the species identification and sexual attractiveness of the trail-producer (Ford, 1978, 1982; Ford & Schofield, 1984). Though there is clear behavioral evidence for a trailing pheromone produced and expressed by females, the chemical structure of such a pheromone has yet to be identified.

Here we report a study designed to evaluate the role of the sexual attractiveness pheromone, a previously characterized sex pheromone produced by female garter snakes, in mediating male reproductive trailing behavior. Composed of a homologous series of long-chain saturated and monounsaturated methyl ketones, this pheromone is contained within the skin lipids of attractive females during the breeding season (Mason *et al.*, 1989; Mason *et al.*, 1990). When a male comes in contact with a female expressing this pheromone, the male initiates courtship behavior characterized by increased tongue-flick rate, chin-rubbing along the dorsum of the female and alignment of cloacal openings (Noble, 1937). In addition to its role in eliciting male courtship behavior, it has been hypothesized that the sexual attractiveness pheromone, when laid down with skin lipids on the substrate as a female passes, is the chemical cue utilized by males to follow the trails of attractive females (Noble, 1937; Garstka & Crews, 1981; Mason, 1992).

The red-sided garter snake, *Thamnophis sirtalis parietalis*, provides an ideal system with which to test this hypothesis. A small subset of males, termed 'she-males', exist in several Manitoba, Canada populations of this species (Mason & Crews, 1985; Shine *et al.*, 2000). She-males are morphologically and anatomically indistinguishable from other males in the population except that they express the sexual attractiveness pheromone on their dorsal surface during the breeding season (Mason, 1993). As a result, other males actively court these she-males as if they were females (Mason &

Crews, 1985). It is not known, however, whether males are capable of trailing she-males during the breeding season. We predict that if the sexual attractiveness pheromone represents the chemical cue utilized by male garter snakes for seeking out potential mates, then male red-sided garter snakes should detect and follow she-male trails to the same extent they do female trails during the breeding season while not responding to trails produced by other males.

Materials and Methods

Study Population and Site

All research was conducted at a study site in the Interlake region of Manitoba, Canada (Narcisse Wildlife Management Area – 50°44'N, 97° 34'W). Populations of red-sided garter snakes inhabiting this region over-winter in large, communal dens and mate immediately upon emergence in the spring (Gregory, 1974). The Narcisse Wildlife Management Area contains three dens in close proximity to one another, with each den possessing in excess of twenty thousand red-sided garter snakes during the winter months (R.T. Mason, unpublished data).

The animals used in this study were captured over a one-week period during the breeding season in May of 1998. Adult males (n = 91) were randomly collected from the den site. Only those males that did not elicit courtship from other males in the field were included in this group. Adult females (n = 27) and she-males (n = 24) were collected by randomly selecting mating groups and locating the source of male courtship behavior (Mason & Crews, 1985). To reduce the error in identifying she-males, we moved a

suspected she-male to a new location at the den site and released it. If the release resulted in the immediate formation of a new mating group centered on the suspected she-male, then we classified the male as a she-male for use in the study.

Behavioral Trials

We utilized a standard Y-maze to test the trailing response of male red-sided garter snakes (*e.g.*, Ford, 1982; Burger, 1989; Figure 3.1a). The maze was constructed of wood and consisted of an initial base arm 1 m in length and 14 cm in width. Joined to the base arm at a Y-junction were two arms of similar dimensions to the base arm separated by a 45° angle. Two rows of vertical pegs were inserted in the arms of the maze to supply traction for the snakes. We covered the surface of the maze with butcher paper and the vertical pegs with drinking straws, all of which were replaced after each trial. To

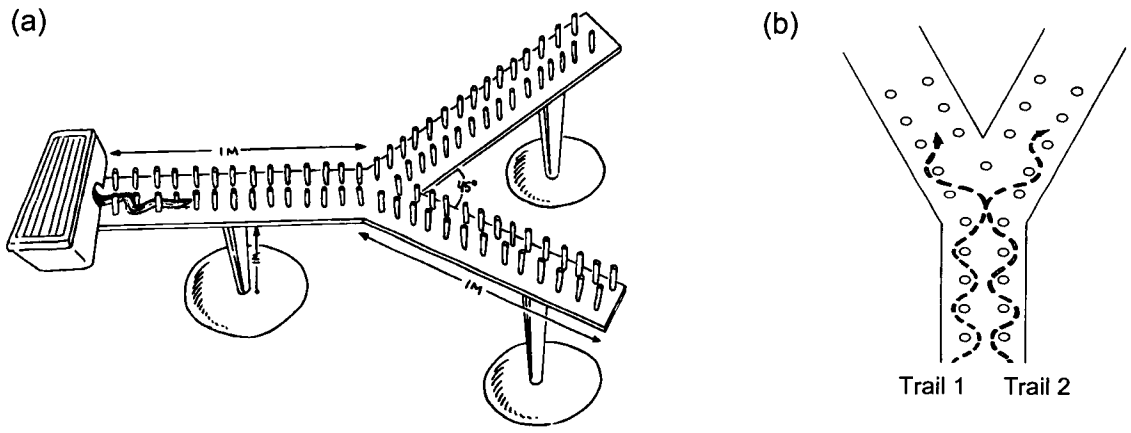

Figure 3.1. Diagram of experimental Y-maze used for trailing experiments (a). When two trails were laid down simultaneously on the Y-maze, they were crossed over one another on the base arm near the Y-junction (b). This allowed for males following one trail to have an opportunity to encounter the second trail before having to choose at the Y-junction.

discourage snakes from exiting the maze, we elevated the maze 1 m off the ground to eliminate the potential for thigmotactic (wall-following) behavior sometimes observed in snakes during similar trailing experiments (*e.g.*, Ford, 1986; Constanzo, 1989).

We performed four experiments testing the ability of male red-sided garter snakes to follow skin lipid trails on the Y-maze. The first two experiments tested the ability of males to follow male trails versus no trail (n = 15 trials) and female trails versus no trail (n = 15 trials). The final two experiments examined the response of male red-sided garter snakes to she-male trails. We tested the response of males to she-male trails versus no trail (n = 12 trials) and the response of males to she-male trails versus female trails (n = 12 trials). The last experiment (she-male trail vs. female trail) was performed to determine whether males could discriminate between the two trail types. We also performed a control experiment (n = 10 trials) at the onset of the study to verify that there was no bias in arm choice by males when no stimuli were present (both arms of the maze blank). Snakes collected in the field were randomly assigned to each experimental group and were used only once before being returned to the site of capture. Trials were conducted daily between the hours of 1000 to 1500, when the snakes were normally active in the field (M.P. LeMaster, personal observation) and environmental conditions (*e.g.*, temperature, light) remained constant throughout the experimental period.

Trails were produced by randomly selecting a stimulus snake (male, female or she-male depending on the experiment) and rubbing the dorsal surface of the snake on the paper and the lower half of the straws covering the maze and pegs. Trails were laid down in this manner from the start of the base arm and continued up the maze to the Y-junction

and then out the entire length of a randomly chosen arm. The cloacal openings of the stimulus animals were covered with tape so that contamination from cloacal gland secretions did not occur. For the experiment testing the ability of males to discriminate between she-male trails and the female trails, trails were placed side-by-side on the base arm and then crossed over each other at the Y-junction before exiting out separate, randomly chosen arms (Figure 3.1b). Trails were crossed at the Y-junction so that a male would have the opportunity to encounter both trails before exiting out one of the arms.

To begin a trial, we placed a male test snake into an opaque box at the start of the base arm and allowed it to acclimate for five minutes. The opening onto the maze was then uncovered and the test snake was allowed to enter the maze of its own accord. Trials ended when the test snake completely entered one exit arm of the Y-maze. Trials in which the test snake failed to exit the hide box after ten minutes or trials in which the test snake fell from the maze were eliminated (< 5% of trials performed). For all successful trials, we recorded the arm chosen by the test snake. We also noted whether the test snake displayed a trail contact response (TCR), an overt response characterized by a snake placing its chin in contact with the substrate and investigating with short, rapid tongue-flicks followed by subsequent movement along the pre-existing trail (Brown & MacLean, 1983).

Statistics

Statistical significance in the trailing response of males for each experiment was examined through Chi-squared tests with a continuity correction (Sokal & Rohlf, 1990). Level of significance was set at $P < 0.05$.

Results

When presented with the control maze (both arms blank), male red-sided garter snakes showed no preference for one arm over the other ($\chi^2 = 0.20$; $P = 0.655$; Table 3.1). Males responded significantly to female trails, choosing the arm with the female trail over the blank arm in all trials conducted ($\chi^2 = 26.13$; $P < 0.001$; Table 3.1). All test males also displayed trail contact responses (TCRs) immediately upon first exiting the start box when tested on female trails. Males did not, however, appear to recognize male trails ($\chi^2 = 0.53$; $P = 0.655$; Table 3.1), and no TCRs were observed during these trials.

Males responded significantly to she-male trails, exiting out the arm of the maze with the she-male trail in 11 of the 12 trials conducted ($\chi^2 = 13.50$; $P < 0.001$; Table 3.1). All test males displayed TCRs, with the lone male not exiting out the she-male trail arm appearing to lose the trail as he approached the Y-junction. Males did not, however, show a preference when presented with a she-male and female trail ($\chi^2 = 0.17$; $P = 0.683$; Table 3.1). All test males did display TCRs in these trials, demonstrating that they were actively trailing, but did not appear to differentiate between the she-male and female trails when having to choose at the Y-junction.

Discussion

Results from this study confirm our initial predictions regarding the response of male red-sided garter snakes to female, she-male and male trails during the breeding season. Male snakes were observed to follow female and she-male trails when presented on the Y-maze but did not show an overt response to male trails. In addition, males were unable to discriminate between female and she-male trails when presented in unison.

Table 3.1. Responses of male red-sided garter snakes, *Thamnophis sirtalis parietalis*, to male, female and she-male trails when tested on a Y-maze (NS = not significant).

Condition	N	Arm Treatment	Number of times selected	P (χ^2)
Control	10	Blank	6	0.655 (NS)
		Blank	4	
Males tested on male trail	15	Male skin lipids	9	0.465 (NS)
		Blank	6	
Males tested on female trail	15	Female skin lipids	15	< 0.001
		Blank	0	
Males tested on she-male trail	12	She-male skin lipids	11	< 0.001
		Blank	1	
Males tested on she-male *versus* female trail	12	Female skin lipids	5	0.683 (NS)
		She-male skin lipids	7	

The sex-specific response of male red-sided garter snakes to female and male trails in this study is concordant with the hypothesis that the sexual attractiveness pheromone is responsible for mediating male trailing behavior in this species. Males responded to female skin rubbing trails, which contain the sexual attractiveness pheromone, but did not respond to male skin rubbing trails, which do not contain the pheromone (Mason *et al.*, 1989). These results confirm prior investigations of garter snake trailing behavior that demonstrate a similar sex-specific trailing response by male snakes (Noble, 1937; Ford, 1982; Ford & O'Blenesss, 1986). The ability of male garter snakes to detect and follow female trails is believed to be an adaptation that allows males, limited by auditory and visual cues, to locate potential mates over long distances (Ford, 1986).

The response of males when presented with she-male trails provides the more compelling evidence that the sexual attractiveness pheromone is the chemical cue utilized by males to follow female trails. The response of males to isolated she-male trails demonstrates that the trailing pheromone is sequestered on the skin surface of she-males, similar to the observations with females. Likewise, behavioral studies reveal that the sexual attractiveness pheromone is present on the dorsal surface of she-males and females during the breeding season (Mason & Crews, 1985; Shine *et al.*, 2000). Furthermore, the inability of males to discriminate between female and she-male trails when presented simultaneously suggests the presence of a pheromone of similar composition; otherwise we would expect males to possess the ability to discriminate among the two trail types owing to the strong selective pressure to locate females, and not she-males, during the breeding season. Chemical analyses of female and she-male skin lipid extracts collected during the breeding season reveal a similar pheromone profile, one that is not observed in the skin lipids of males collected during the same time period (Mason, 1993).

Although the results of this study support the hypothesis that the sexual attractiveness pheromone mediates male reproductive trailing behavior in the red-sided garter snake, we acknowledge that alternative hypotheses do exist. For example, the pheromone responsible for eliciting male trailing behavior in this species may be a novel compound, or suite of compounds, contained in the skin lipids of females and she-males, but not males, which has yet to be identified. Future research is necessary to determine whether male garter snakes are capable of detecting and following trails consisting solely of the sexual attractiveness pheromone when isolated from the skin lipids of attractive females and she-males.

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Chapter 4

Annual and Seasonal Variation in the Female Sexual Attractiveness Pheromone of the Red-sided Garter Snake, *Thamnophis sirtalis parietalis*

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Submitted to *Chemical Signals in Vertebrates IX*,
Plenum Press, New York, New York
July 2000, 8 pages, *in press*.

Abstract

Female garter snakes express a sexual attractiveness pheromone, composed of saturated and unsaturated methyl ketones, that is necessary to elicit male courtship behavior during the breeding season. Because garter snake reproductive success depends on the female sexual attractiveness pheromone, it might be expected that the composition of this pheromone would remain relatively stable across breeding seasons. Additionally, it might be expected that the expression of this pheromone would vary between the breeding season and non-breeding season, functioning as a mechanism for regulating male courtship behavior. Here, we present a study designed to examine annual and seasonal variation in the composition of the female sexual attractiveness pheromone of the red-sided garter snake, *Thamnophis sirtalis parietalis*. To evaluate annual variation, pheromone samples were collected from groups of sexually attractive females upon spring emergence from underground hibernacula in Manitoba, Canada, during three consecutive years. To evaluate seasonal variation, samples were collected from a group of attractive females upon spring emergence and a group of unattractive females gathered at the hibernacula in the autumn of a single year. Annual samples showed little variation in the relative concentrations of saturated and unsaturated methyl ketones across years suggesting that the composition of the female sexual attractiveness pheromone is tightly regulated across breeding seasons for this species. Seasonal samples showed a marked difference in the relative concentrations of saturated and unsaturated methyl ketones, indicating that the composition of this pheromone fluctuates between the breeding and non-breeding season and may represent a mechanism for regulating male courtship behavior between the seasons.

Introduction

Reptiles are excellent models for vertebrate pheromone research because the initiation of many behaviors is dependent on pheromone production and expression (reviewed in Madison, 1977; Mason, 1992; Mason *et al.*, 1998). For example, the reproductive success of snakes depends on the production and perception of specific sex pheromones (Noble, 1937; Gillingham & Dickinson, 1980; Andrén, 1986; Mason *et al.*, 1989). In the absence of these pheromones, sexual behavior will not be initiated and mating will not occur.

Garter snakes (genus *Thamnophis*) are one of the most commonly encountered snakes in North America with a range stretching from central Canada to Costa Rica (Rossman *et al.*, 1996). Since the 1930's, investigators have demonstrated that within this genus initiation of male courtship behavior depends on the production and expression of a female sexual attractiveness pheromone (*e.g.,* Noble, 1937; Garstka & Crews, 1981; Mason *et al.*, 1989). When a male comes in contact with a female expressing such a pheromone, the male displays courtship behavior characterized by increased tongue-flick rate, chin-rubbing along the dorsum of the female and alignment of cloacal openings (Noble, 1937). These behaviors are simultaneously expressed only in a reproductive context in response to the sexual attractiveness pheromone (Camazine *et al.*, 1980).

The sexual attractiveness pheromone of the red-sided garter snake *(Thamnophis sirtalis parietalis*) is the first, and to date only, reptilian sex pheromone isolated and identified. The female sexual attractiveness pheromone of this species consists of a homologous series of long-chain saturated and ω–9 cis-unsaturated methyl ketones contained within the skin lipids of the female (Mason *et al.*, 1989; Mason *et al.*, 1990).

Although both the saturated and unsaturated methyl ketones are required to elicit full male courtship behavior, unsaturated methyl ketones appear to be the more biologically active of the two groups. When presented in isolation, unsaturated methyl ketones elicit a five-fold increase in male response over saturated methyl ketones (Mason *et al.*, 1989).

As part of our laboratory's ongoing work towards deciphering the pheromone system of the red-sided garter snake, we present here a study examining annual and seasonal variation in the composition of the female sexual attractiveness pheromone for this species. Because garter snake reproductive success depends on the production and expression of the female sexual attractiveness pheromone, we predict that the composition of this pheromone will remain relatively stable across breeding seasons for the red-sided garter snake. Additionally, we predict that there will exist observable differences in the composition of this pheromone between the breeding season and the non-breeding season, representing a mechanism for regulating male courtship behavior out of the breeding season. Currently, there is limited information available concerning temporal variation in the production and expression of vertebrate reproductive pheromones.

Materials and Methods

Study Population

The red-sided garter snake (*Thamnophis sirtalis parietalis*) is the most northerly living reptile in the Western Hemisphere (Logier & Toner, 1961). The annual aggregation of red-sided garter snakes at underground hibernacula in Manitoba, Canada

is a unique natural phenomena representing the highest concentration of snakes in the world (Gregory, 1984). Marshes, shallow lakes and poor drainage offer good summer feeding grounds for the snakes while limestone bedrock provides hibernaculum sites where snakes are constrained to spend up to eight months of the year to avoid the harsh winters (Aleksiuk & Stewart, 1971).

Red-sided garter snakes utilized for this study were obtained from a field site at the Narcisse Wildlife Management Area in the Interlake region of Manitoba, Canada (50°44'N, 97°34'W). The Narcisse Wildlife Area contains three over-wintering hibernacula in close proximity to each other, with each hibernaculum possessing in excess of twenty thousand red-sided garter snakes during the winter months (R.T. Mason, unpublished data).

Skin Lipid Collection and Fractionation

For the annual variation samples, adult, sexually attractive female red-sided garter snakes were randomly collected immediately following spring emergence from the hibernacula in May of 1997 (n = 19; snout-vent length (SVL) = 62.2 (± 8.1) cm; mass = 93.6 (± 41.8) g), 1998 (n = 20; SVL = 62.7 (± 6.7) cm; mass = 93.0 (± 31.1) g), and 1999 (n = 12; SVL = 65.7 (± 4.5) cm; mass = 103.4 (± 19.6) g). For the seasonal variation samples, adult, attractive female red-sided garter snakes were randomly collected from the hibernacula in May 1997 (n = 5; SVL = 58.3 (± 4.1) cm; mass = 72.1 (± 13.6) g) and adult, unattractive females were randomly collected from the hibernacula in September 1997 (n = 5; SVL = 57.8 (± 4.2) cm; mass = 74.2 (± 16.0) g).

Following each sampling period, the animals were briefly washed (1 minute per

snake) in 300 ml hexane (C_6H_{14}) to remove a pooled sample of their skin lipids. The excess solvent was then evaporated off the skin lipid extract and the resulting residue was resuspended in fresh hexane and sealed in a 9-ml glass vial with a polyethylene-lined cap for storage at –20°C. The animals, following a brief recovery at the Chatfield research station (Chatfield, Manitoba) were returned to the point of capture.

Fractionation of the extracted skin lipids was accomplished in the laboratory using alumina activity III columns with hexane and ether as the mobile phases (Mason *et al.*, 1989). For each sample, the fraction containing the methyl ketones composing the female sexual attractiveness pheromone was removed (fraction 5 - Mason *et al.*, 1989) and the excess solvent evaporated off. The remaining residue was then resuspended in fresh hexane and sealed in a 9-ml glass vial with a polyethylene-lined cap for storage at -20°C.

Chemical Analysis

Analysis of the methyl ketones composing the sexual attractiveness pheromone was conducted on a Hewlett Packard 5890 Series II gas chromatograph fitted with a split injector (280°C) and a Hewlett Packard 5971 Series mass selective detector. A fused-silica capillary column (HP-1; 12 m x 0.22 mm ID; Hewlett Packard, California, USA) was used with helium as the carrier gas (5 cm/sec). All injections were made in the splitless mode with the split valve closed for 60 sec. Oven temperature was initially held at 70°C for 1 min, increased to 210°C at 30°C/min, held at 210°C for 1 min, increased to 310°C at 5°C/min, and held at 310°C for 5 min.

Quantification of the saturated and unsaturated methyl ketones was accomplished using peak integration to calculate the percent relative concentrations of each to the overall pheromone profile for a sampling period (*e.g.*, percent relative concentration of saturated methyl ketones = area under saturated methyl ketone peaks / total area of all methyl ketone peaks x 100). Retention times and peak areas were determined using ChemStation software (Version B.02.05; Hewlett Packard, California, USA) interfaced with the gas chromatograph/mass spectrometer (GC/MS).

Results

Annual Variation

When compared across years, there was no difference in the average snout-vent length (ANOVA, $F = 1.030$, $P > 0.25$) or mass (ANOVA, $F = 0.416$, $P > 0.50$) of females sampled.

Complete GC/MS analysis of the methyl ketone fractions from the three years revealed the presence of eighteen unique long-chain methyl ketones. Nine of these were identified as saturated methyl ketones while the other nine had mass spectra in accord with unsaturated methyl ketones (Mason *et al.*, 1990). Overall, there were only minor differences observed in the percent relative contribution of saturated and unsaturated methyl ketones to the overall pheromone profiles across the years (Figure 4.1). In all three years, unsaturated methyl ketones dominated the pheromone profiles, accounting for 81.3% of the pheromone profile in 1997, 82.4 % in 1998 and 86.1 % in 1999.

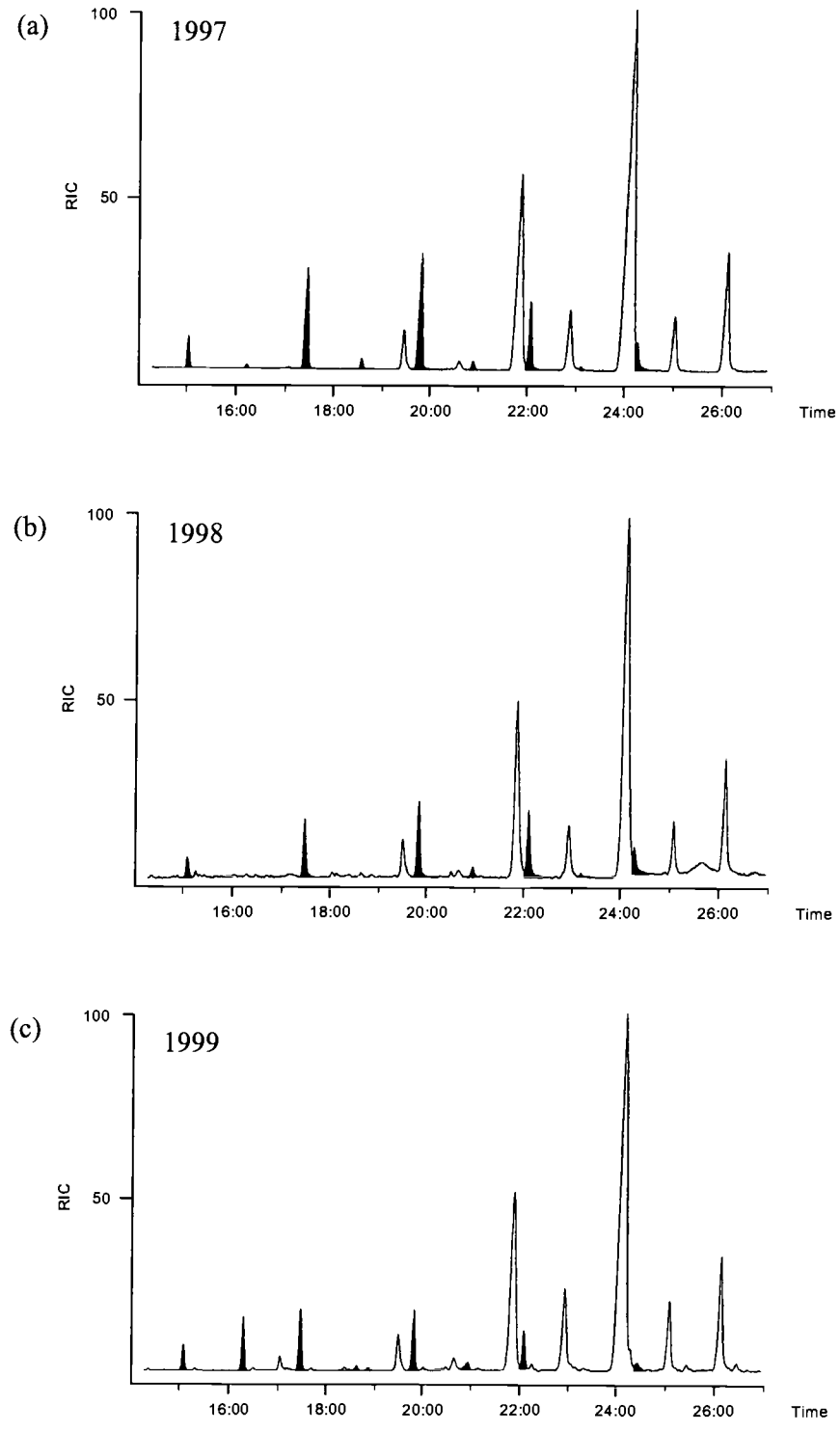

Figure 4.1. Gas chromatograms of the female sexual attractiveness pheromone profile for red-sided garter snakes sampled in (a) 1997, (b) 1998 and (c) 1999. Pheromone profiles are composed of saturated (shaded peaks) and unsaturated (open peaks) methyl ketones.

Seasonal Variation

When compared across seasons, there was no difference in the average snout-vent length (ANOVA, $F = 0.042$, $P > 0.50$) or mass (ANOVA, $F = 0.052$, $P > 0.50$) of females sampled.

Complete GC/MS analysis of the methyl ketone fractions from the two seasons revealed the presence of the eighteen unique long-chain methyl ketones previously described. Overall, there was a distinct difference in the percent relative concentrations of saturated and unsaturated methyl ketones composing the pheromone between seasons (Figure 4.2). Unlike the spring sample, which displayed a profile rich in unsaturated methyl ketones (72.0%), the fall sample shows a marked decrease in the percent relative concentration of unsaturated methyl ketones (63.0%).

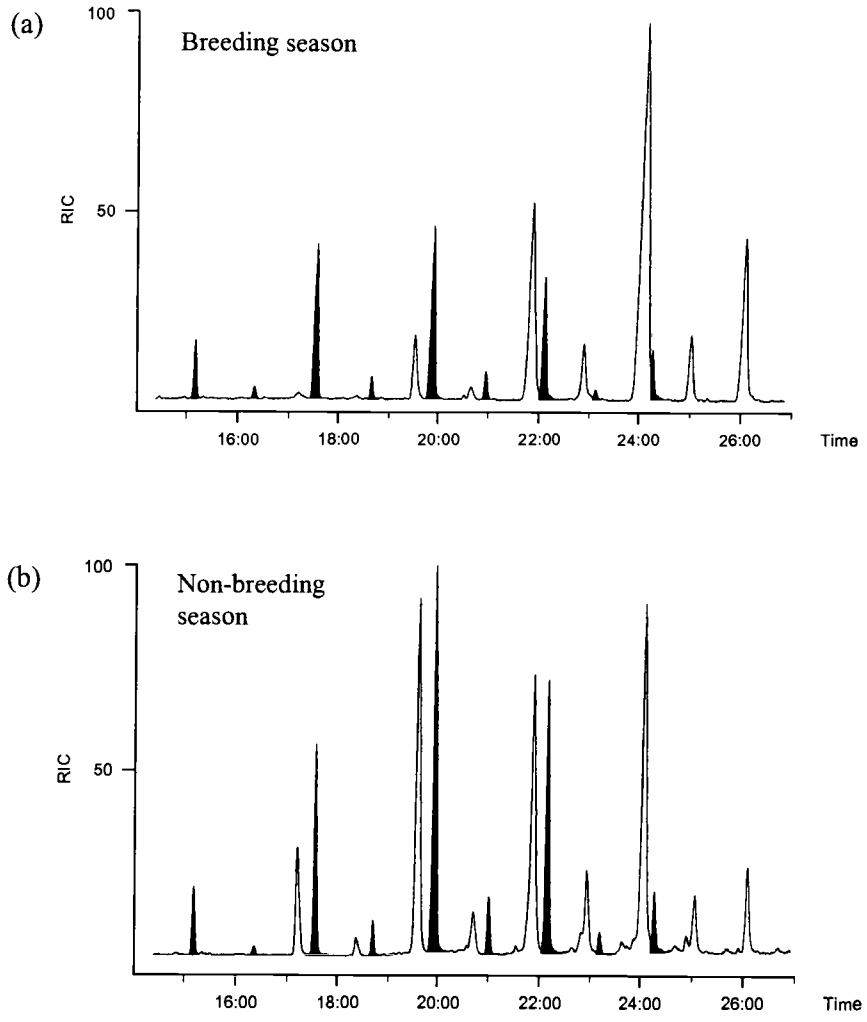

Figure 4.2. Gas chromatograms of the female sexual attractiveness pheromone profile for red-sided garter snakes sampled during (a) the breeding season and (b) the non-breeding season. Pheromone profiles are composed of saturated (shaded peaks) and unsaturated (open peaks) methyl ketones.

Discussion

The results of this study demonstrate that the composition of the female sexual attractiveness pheromone does exhibit temporal variation. As predicted, complete GC/MS analysis revealed only minor fluctuations in the relative concentrations of saturated and monounsaturated methyl ketones to the overall pheromone profiles across years, but revealed distinct differences in the pheromone profiles between the seasons. To the best of our knowledge, this is the first study to examine annual and seasonal variation in the expression of a characterized vertebrate pheromone.

The lack of variation across the three years of this study suggests that the composition of the female sexual attractiveness pheromone is tightly regulated during the breeding season. It is not surprising that some degree of regulation was observed. Communication systems depend on the production of a signal by the signaler and the perception of the signal by a receiver. If the variation of a signal extends beyond the range of what the receiver is tuned for, then the signal will no longer function as intended (Bradbury & Vehrencamp, 1998). Thus, female garter snakes expressing methyl ketone profiles that fall out of the tuning range of males would not elicit courtship behavior nor successfully mate.

Insects have been noted to display precise control over sex pheromone expression (*e.g.*, Miller & Roelofs, 1980), similar to our observations. This precise regulation is often regarded as a mechanism by which closely related species, living in sympatry and utilizing similar pheromone components, can remain reproductively isolated (Cardé & Baker, 1984; Attygalle *et al.*, 1986). The red-sided garter snake lives in sympatry with a closely related species, the plains garter snake (*Thamnophis radix*), in Manitoba, Canada.

Initial examinations of the methyl ketone profile for the plains garter snake reveals a profile similar in composition, but distinct in the relative concentrations of individual components, to that for the red-sided garter snake (R.T. Mason, unpublished data). Future studies will examine the role of the female sexual attractiveness pheromone in the reproductive isolation of these two species.

The observed variation in the pheromone profiles between the breeding season and the non-breeding season suggests that the integrity of the female sexual attractiveness pheromone of the red-sided garter snake is not maintained throughout the year. Most likely, females halt production of the pheromone following the breeding season, similar to what occurs in most insects expressing sex pheromones contained within the cuticular skin lipids during the breeding season (reviewed in Tillman *et al.*, 1999). The change in composition observed in the garter snake may result from differences in the production rates of the saturated and unsaturated methyl ketones. Unlike unsaturated methyl ketones, saturated methyl ketones are present in the skin lipids of males as well as females (Mason, 1993) suggesting a possible biological function other than sex recognition. Thus, females most likely shift the levels of unsaturated methyl ketones, the more active of the two pheromone components (Mason *et al.*, 1989), between the breeding and non-breeding season while maintaining a similar saturated methyl ketone level. Although the exact physiological mechanisms involved in regulating pheromone production in garter snakes are not known, the expression of the sexual attractiveness pheromone appears to be under endocrine regulation. Estrogen treatment has been shown to induce pheromone production in female garter snakes (Crews, 1976; Kubie *et al.*, 1978; Garstka & Crews, 1981; Crews, 1985).

Because male red-sided garter snakes have an obligate reliance on the pheromone to initiate courtship behavior, this seasonal shift in pheromone composition may explain the lack of male courtship interest displayed to females during the non-breeding season. Insects often rely on shifts in pheromone composition to regulate courtship behavior (*e.g.,* Schal *et al.*, 1994; Hurd & Perry, 1991). In support of this hypothesis is the fact that a small subset of female garter snakes do elicit male courtship behavior out of the breeding season (M.P. LeMaster, personal observation). This observation indicates that males are still receptive to the pheromone outside the breeding season, unlike males of other vertebrate species which may be physiologically unreceptive to pheromone cues in the non-breeding season (*e.g.,* newts, Moore *et al.*, 2000). Additional studies are necessary to demonstrate whether the small subset of females attracting courtship attention outside the breeding season display a pheromone profile similar to that observed in the breeding season, or whether there are some other mechanisms at work which still remain to be elucidated.

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Chapter 5

Pheromonal Mediation of Mate Choice in the Red-sided Garter Snake, *Thamnophis sirtalis parietalis*

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Submitted to *Journal of Chemical Ecology*,
Plenum Press, New York, New York
August 2001, 31 pages, *in review*.

Abstract

Male red-sided garter snakes (*Thamnophis sirtalis parietalis*) display a courtship preference for larger females during the breeding season. Utilizing behavioral experiments and chemical analyses, we tested the hypothesis that males discriminate among females of varying size by means of the sexual attractiveness pheromone, a previously characterized sex pheromone composed of a homologous series of long-chain saturated and ω-9 cis-unsaturated methyl ketones contained in the skin lipids of females. When presented with skin lipid extracts from large and small females, a greater proportion of males preferred to display courtship behaviors to large female extracts, demonstrating that size preference is mediated through chemical cues contained within the skin lipids. Analysis of the sexual attractiveness pheromone revealed the necessary variation for this pheromone to function as a reliable indicator to males of female body size. Specifically, we observed a strong correlation between female snout-vent length and the relative concentration of saturated and ω-9 cis-unsaturated methyl ketones composing the pheromone; smaller females expressed pheromone profiles higher in saturated methyl ketones while larger females expressed pheromone profiles dominated by unsaturated methyl ketones. These results support the hypothesis that the sexual attractiveness pheromone represents the cue used by male red-sided garter snakes to differentiate among potential mates of varying size.

Introduction

In many vertebrate species, individuals of one sex or both sexes actively select among potential mates during the breeding season to enhance their reproductive success

(Andersson, 1994). Individuals often discriminate among potential mates using variation in the expression of a particular morphological trait. For example, females of many bird species differentiate among males using visual traits, (*e.g.*, color intensity – Hill, 1990; tail length – Møller, 1988) whereas many female frogs utilize variation in auditory traits (*e.g.*, call rate – Sullivan, 1983; call pitch – Ryan, 1980). Chemical traits, such as pheromones (Karlson & Lüscher, 1959), represent an additional type of trait by which potential mates might be appraised.

The occurrence of chemically mediated mate choice is relatively common in insects in which chemical communication is considered the dominant modality for controlling reproductive behavior in many species (Bell & Cardé, 1984). However, few studies have documented chemically mediated mate choice in vertebrates (*e.g.*, Verrell, 1985; Reece-Engel, 1988; Martín & López, 2000). This paucity of studies is primarily due to the multicomponent sensory nature of vertebrates where a combination of sensory inputs (*e.g.*, visual, tactile, chemical) are often responsible for mediating a particular behavior (Albone, 1984). Such diversification makes it difficult to establish effective bioassays for measuring behavioral responses of individuals to isolated chemical cues in order to determine to what extent such cues are utilized.

Snakes offer an excellent model system in which to investigate the chemical mediation of mate choice in vertebrates. More than most vertebrates, snakes rely extensively upon the production and perception of specific sex pheromones for the coordination of reproductive behavior (reviewed in Mason, 1992; Mason *et al.*, 1998). Hence, there is an increased probability that mechanisms evolved to select among potential mates (mate choice in snakes - Hawley & Aleksiuk, 1976; Luiselli, 1996) will

be chemical in nature. In addition, snakes are much like insects in that they respond to isolated chemical cues with stereotypical, robust behaviors (Brown & MacLean, 1983; Mason & Crews, 1985). Thus, behavioral bioassays can be constructed with relative ease to test the responses of snakes to chemical cues from potential mates when presented in isolation.

Annual aggregations of red-sided garter snakes (*Thamnophis sirtalis parietalis*) at underground hibernacula in Manitoba, Canada are unique natural phenomena representing the highest concentrations of snakes in the world (Gregory, 1984). Mating in these populations occurs directly at the hibernacula following spring emergence with virtually all females (> 95% - Garstka *et al.*, 1982) mating within one day of emerging from winter dormancy. During this period, it is not uncommon to observe 10-100 males courting a newly emerged female, forming what are termed 'mating balls' (Gregory, 1974, 1977). Males do not randomly associate with females, but instead display a courtship preference for larger females (Hawley & Aleksiuk, 1976; Shine *et al.*, 2001).

It has been hypothesized that the cue utilized by male red-sided garter snakes to differentiate among potential mates is the sexual attractiveness pheromone (Garstka *et al.*, 1982; Gregory, 1984; Mason, 1992). Composed of a homologous series of saturated and ω-9 cis-unsaturated methyl ketones (Mason *et al.*, 1989; Mason *et al.*, 1990), this pheromone is sequestered in the skin lipids of females during the breeding season and is primarily responsible for eliciting male courtship behavior (Noble, 1937; Garstka *et al.*, 1982). Indeed, if a male does not detect this pheromone on the dorsal surface of a female then courtship behavior will not be initiated and mating will not occur (Mason, 1993). Thus, variation in the expression of the sexual attractiveness pheromone by females of

varying size may directly influence male courtship behavior, leading to the mating preference observed in this species.

Here we report a study designed to investigate the role of the female sexual attractiveness pheromone in mediating male mate choice in the red-sided garter snake. Our two major aims in this study are 1) to determine whether males can discriminate among females of varying size based solely on chemical cues in the skin lipids and 2) to evaluate whether the sexual attractiveness pheromone contains the necessary variation to function in male mate choice. To this end, we conducted behavioral experiments testing the courtship response of males to skin lipid extracts from large and small females. Further, we collected pheromone samples from females of varying size and examined the quantity and quality of pheromone expressed by individual females.

Materials and Methods

Study Population

Red-sided garter snakes utilized for this study were captured at a field site near the community of Inwood in the Interlake region of Manitoba, Canada (50°31.58'N; 97°29.71'W) during May of 2000. This site is located at an abandoned gravel quarry containing a single over-wintering hibernaculum possessing in excess of ten thousand red-sided garter snakes during the winter months (R.T. Mason, unpublished data). Adult females in this population attain an average snout-vent length (SVL) of 55-60 cm and a mean mass of 70-85 g while adult males are much smaller, attaining an average SVL of 45-50 cm and a mean mass of 35-40 g (Shine *et al.*, 1999).

Behavioral Experiments

Experimental Animals

Unmated large female adult red-sided garter snakes (SVL > 60.0 cm; n = 35) and small female adult red-sided garter snakes (SVL < 50.0 cm; n = 35) were collected at the hibernaculum immediately upon emergence. We established the SVL criteria for large and small females based on the results of Hawley and Aleksiuk (1976), who found that female red-sided garter snakes with SVL > 60 cm were twice as likely to be courted by males than females with SVL < 50 cm. Adult males (n = 380; SVL (± SD) = 52.4 (± 2.54)) were collected from naturally occurring mating balls located around the hibernacula. We focused our collection on larger males (SVL > 50.0 cm) as larger males show an increased ability to differentiate between females based on size than do smaller males in this species (Shine *et al.*, 2001). Animals were segregated by sex and size in cloth bags and held at ambient temperatures until testing, after which the animals were returned to the hibernaculum and released.

Testing Conditions

Behavioral experiments were carried out in outdoor arenas measuring 1 x 1 x 1 m and constructed of nylon fabric attached to metal posts inserted in the ground. Arenas of this type do not appear to alter male reproductive behaviors compared to the behaviors observed in the wild and as a result such arenas have been used extensively in the study of snake reproductive ecology (*e.g.*, Mason & Crews, 1985; Shine *et al.*, 1999; Moore *et al.*, 2000). To avoid any confounding effects due to weather conditions, an array of 4

arenas was established allowing for groups of trials to be carried out simultaneously. Testing days and times of day (1000 hrs to 1600 hrs) were chosen so that all experiments were conducted under similar ambient conditions (mostly sunny skies with light winds and temperatures of 15-20°C) which correspond with conditions when red-sided garter snakes are most active (M.P. LeMaster, personal observation).

Experiment 1:

To verify that males in our study population exhibit a courtship preference for larger females, we first tested the behavioral response of males to large and small females using a simultaneous choice test design (*e.g.*, Mason & Crews, 1985). Briefly, we introduced ten randomly chosen males into an arena and allowed them to acclimate for five minutes. We then placed into each arena simultaneously a large and small female. The snakes were allowed to interact undisturbed for five minutes after which time the number of males actively courting each female size was recorded. A total of sixteen tests were performed with unique male and female snakes used in each trial.

Male courtship behavior was assessed using an ethogram of male garter snake mating behavior (Table 5.1). Similar ethograms are routinely used in field and laboratory studies with garter snakes (*e.g.*, Crews *et al.*, 1984; Moore *et al.*, 2000). For a male to be considered actively displaying courtship behavior during this study, a score of 2.0 or greater was required on the ethogram. To prevent females from mating during the trials, an event that drastically reduces further male courtship advances (Garstka *et al.*, 1982), we placed adhesive tape across the female cloaca that was removed immediately upon completion of each trial.

Table 5.1. Ethogram of courtship behavior for the male red-sided garter snake, *Thamnophis sirtalis parietalis* (Modified from Crews *et al.*, 1984). A behavioral score of 2.0 or greater only occurs in a reproductive context and is therefore indicative of sexual behavior for males of this species.

Courtship Score	Description of Behavior
1.0	Male investigates female with increasing tongue-flick rate
2.0	Male chin-rubs dorsal surface of female and aligns body with female
3.0	Male actively tail searches and attempts cloacal apposition with female
4.0	Male copulates with female

Experiment 2:

This experiment utilized behavioral trials to determine whether chemical cues contained within the skin lipids of females are sufficient for male mate discrimination. For each trial, we introduced ten randomly chosen males into an arena and allowed them to acclimate for five minutes. We then randomly placed one of four treatments in the arena: 1) a small female, 2) a large female, 3) filter paper containing a small female skin lipid sample or 4) filter paper containing a large female skin lipid sample. Male snakes were allowed to interact with the female or filter paper undisturbed for five minutes after which time the number of males actively courting (ethogram score of 2.0 or greater) the treatment was recorded. A total of thirty-eight trials were performed using small (n = 9) and large (n = 9) female snakes and skin lipid samples from small (n = 10) and large (n = 10) female snakes. Unique males and females were used in each trial.

Skin lipid samples were collected from large and small females by rubbing the dorsal surface of the donor snake with filter paper wetted with 100% hexane. Care was taken to prevent contamination from cloacal gland secretions (Mason *et al.*, 1989). For each skin lipid sample collected, a unique female was used. To control for surface area

differences in the two groups of females, we rubbed the length of the large females once with the filter paper and rubbed the length of the small females three times. We established this protocol based on prior knowledge that 1) hexane extracts from large females yield three times the amount of skin lipids compared to small females and 2) multiple rubbing of a female does not affect male behavior towards the subsequent skin lipids extracted (M.P. LeMaster, unpublished data). To verify that similar quantities of skin lipids were laid down for each female size, we compared the average skin lipid amount laid down on the filter papers for each female size upon completion of the trials (skin lipids (mg) = weight of treated filter paper – weight of blank filter paper).

Chemical Analysis

Pheromone Collection

Adult, sexually attractive female red-sided garter snakes of varying size (SVL range = 48.0 cm to 74.2 cm; n = 20) were collected immediately upon spring emergence from the hibernaculum. The size range of females collected corresponded with the size range of females used in our behavioral experiments. The animals were killed with an overdose of brevital sodium. Each snake was then placed dorsal side down in a 500-ml glass beaker and covered with 25-50 ml of 100% hexane (C_6H_{12}) for 12 hours (Mason *et al.*, 1989; Mason *et al.*, 1990). Care was taken to keep the head and cloaca out of the hexane to avoid possible contamination by internal bodily fluids. After removal of the animals, the excess solvent was removed under reduced pressure by rotoevaporation at 35°C. The resulting residues were weighed on a digital scale (Mettler AT400),

resuspended in fresh hexane (1-2 ml), and sealed in 9-ml glass vials with polyethylene-lined caps for storage at -20°C.

To isolate the methyl ketones composing the sexual attractiveness pheromone, we fractionated the skin lipid extracts using column chromatography as described by Mason *et al.* (1989). Briefly, we loaded the skin lipid extracts onto glass columns (350 mm x 22 mm ID) packed with alumina (activity III) and eluted the columns with hexane and ethyl ether ($C_4H_{10}O$) solutions of increasing polarity. For each sample, the fraction containing the appropriate methyl ketones (fraction 5 – Mason *et al.*, 1989) was collected and the excess solvent was removed by rotoevaporation (35°C). The resulting methyl ketone residues were weighed on a digital scale (Mettler AT400) and resuspended in fresh hexane (1 ml). Samples were then placed in 9-ml glass vials with polyethylene-lined caps and stored at –20°C until further analysis.

Pheromone Analysis

Multi-component pheromones, such as the sexual attractiveness pheromone of the red-sided garter snake, can show variation in both the quantity and quality of pheromone expressed. To examine variation in the quantity of pheromone expressed by female snakes of varying size, we calculated the amount of methyl ketones expressed per unit skin surface area ($\mu g/cm^2$) for individual females. This was accomplished by dividing the weight of the isolated methyl ketone residues extracted from a female by the total skin surface area of the female. A general measure of skin surface area for each female was determined by multiplying the snout-vent length of a female by its circumference at mid-body (Mason *et al.*, 1990).

To examine variation in the quality of pheromone expressed by females, we determined the number of unique methyl ketones expressed by individual females and calculated the relative contributions of saturated versus monounsaturated methyl ketones to the individual pheromone profiles. The methyl ketones present in the pheromone extracts were identified utilizing a Hewlett Packard 5890 Series II gas chromatograph fitted with a split injector (280°C) and a Hewlett Packard 5971 Series mass selective detector. Aliquots (1 μl) of the methyl ketone fractions were injected onto a fused-silica capillary column (HP-1; 12 m x 0.22 mm ID; Hewlett Packard, California, USA) with helium as the carrier gas (5 cm/sec). Oven temperature was initially held at 70°C for 1 min, increased to 210°C at 30°C/min, held at 210°C for 1 min, increased to 310°C at 5°C/min, and finally held at 310°C for 5 min. Once the methyl ketones were identified, we then calculated the relative concentrations of saturated and monounsaturated methyl ketones in each sample using peak integration (*e.g.*, percent relative concentration of saturated methyl ketones = area under saturated methyl ketone peaks / total area of all methyl ketone peaks x 100). Identification of compounds and peak areas were determined utilizing ChemStation software (Version B.02.05; Hewlett Packard, California, USA) interfaced with the gas chromatograph/mass spectrometer.

Statistics

All statistical analyses were performed using Jandel SigmaStat Version 2.0 software package (Jandel Corporation). The difference in the number of simultaneous choice tests ending with more males courting larger females than smaller females was analyzed using a Wilcoxon sign-rank test. Differences in the proportion of males

courting large and small females and large and small female skin lipid samples were initially analyzed using one-way analysis of variance (ANOVA) with Tukey post-hoc tests then used to perform pair-wise comparisons among the means of the four treatments. Finally, the relationship between the size of the female (snout-vent length) and various quantitative and qualitative measures of pheromone expression were examined utilizing Pearson product moment correlation tests (Sokal & Rohlf, 1995). Level of significance for each test was set at $P < 0.05$.

Results

Behavioral Experiments

Experiment 1

The average SVL (± SD) of small females used in the simultaneous choice tests was 46.2 cm (± 2.70) whereas the average SVL (± SD) for large females was 63.0 cm (± 2.57). This resulted in an average (± SD) difference between small and large females presented to males during individual trials of 16.84 cm (± 3.93). In all sixteen trials conducted, a greater proportion of males were observed courting the larger female over the smaller female following the five minute interaction period (Wilcoxon signed-rank test: $T = 136$, $N = 16$, $P < 0.001$; Figure 5.1). On average across the trials, 64.3% of the males were found courting the larger females whereas only 11.9% of the males were observed actively courting the smaller females.

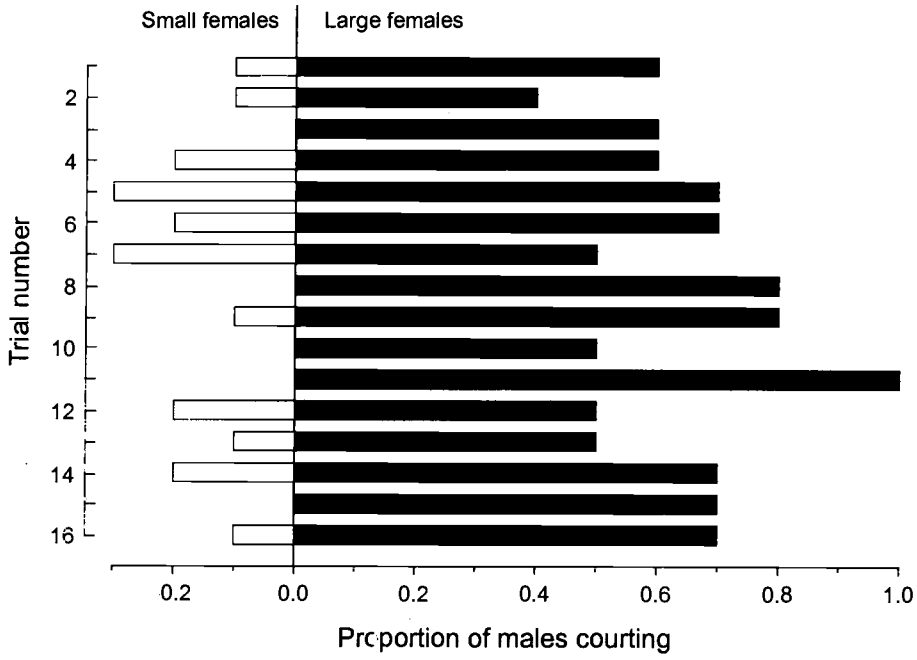


Figure 5.1. Results of simultaneous choice tests in which a large and a small female red sided garter snake (*Thamnophis sirtalis parietalis*) were placed in an outdoor arena with 10 courting males. After five minutes the number of males that courted either female was recorded.

Experiment 2

When presented with the four treatments, male garter snakes responded with stereotypical courtship behaviors including increased tongue-flick rate and chin rubbing along the dorsum of the female or filter paper depending on the treatment. Overall, we observed significant variation in the proportion of males actively courting the various treatments after the five minute interaction period (ANOVA, $F = 18.12$, $P < 0.001$; Figure 5.2). Pair-wise analysis revealed that a significantly greater proportion of males directed courtship behavior to large females and filter paper containing large female skin lipids than to small females and filter paper containing small female skin lipids (Tukey tests; $q = 6.06 - 8.50$; $P < 0.001$ for all tests).

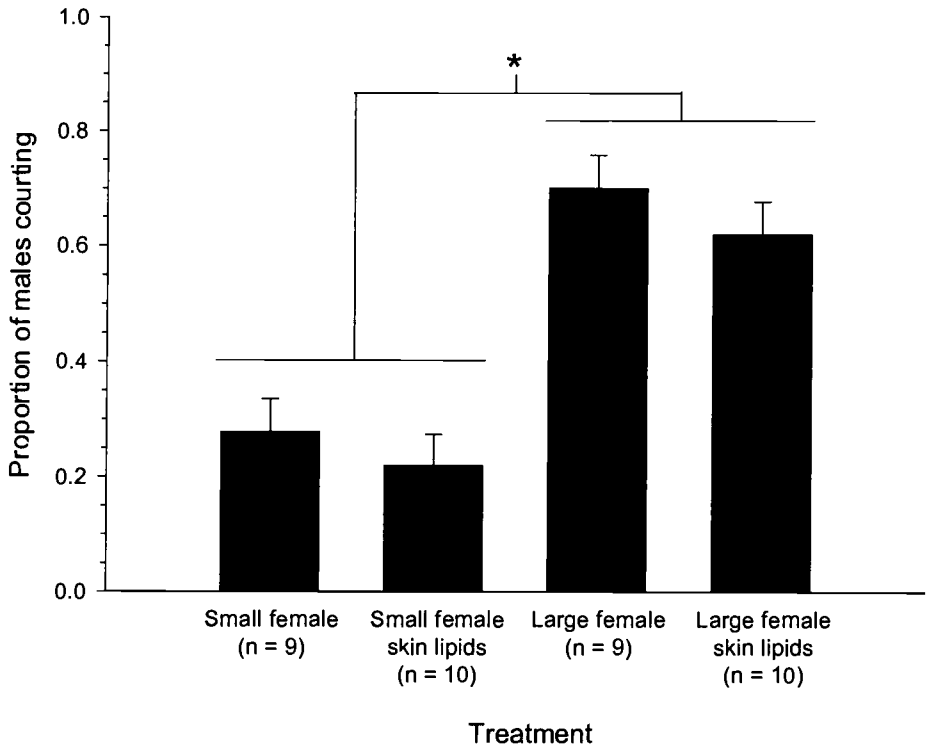


Figure 5.2. Mean (± SE) proportion of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) displaying courtship behavior to large and small females and skin lipid samples from large and small females.

However, little difference was observed in the proportion of males displaying courtship behavior to large females and large female skin lipid extracts (Tukey test; $q = 1.41$; $P = 0.750$) and to small females and small female skin lipid extracts (Tukey test; $q = 1.02$; $P = 0.887$).

When we examined the amount of skin lipids placed on the treated filter papers, we found no difference between filter papers containing large female skin lipid samples and filter papers containing small female skin lipid samples (large female average (± SE) = 3.0 (± 0.8) mg, small female average (± SE) = 3.2 (± 0.6) mg; ANOVA, $F_{1,18} = 0.041$, $P = 0.842$). In addition, there was no difference in snout-vent length (± SD) between

large females presented to males and large female skin lipid donors (test female = 66.1 cm (± 3.63), donor female = 66.7 cm (± 4.66); t-test: t = 0.308, P = 0.762) or between small females and small female skin lipid donors (test female = 46.7 cm (± 4.60), donor female = 44.2 cm (± 3.16); t-test: t = 1.379, P = 0.186).

Pheromone Analysis

Pheromone Quantity

The hexane extractions of individual females yielded an average (± SE) of 21.3 (± 1.7) mg of skin lipids per female. Subsequent fractionation of the lipids yielded an average (± SE) methyl ketone fraction of 1.3 (± 0.2) mg per female. Overall, the methyl ketones accounted for an average (± SE) of 6.1 (± 0.8) % of the skin lipids collected from the female snakes. After accounting for variation in skin surface area, individual females were found to vary widely in the quantity of methyl ketones expressed on their dorsal surface (range = 1.8 $\mu g/cm^2$ to 16.4 $\mu g/cm^2$; Table 5.2). There was not, however, a significant relationship between the female snout-vent length and the amount of methyl ketones extracted per unit of surface area (Pearson product-moment correlation: r = 0.169, N = 20, P = 0.476).

Pheromone Quality

Complete GC-MS analysis of the methyl ketone fractions revealed the presence of eighteen unique long-chained methyl ketones (Figure 5.3). Nine of these were identified

Table 5.2. Quantitative and qualitative variation in expression of the sexual attractiveness pheromone among individual female red-sided garter snakes, *Thamnophis sirtalis parietalis*. Female snakes are arranged according to snout-vent length (small to large).

Individual female	Snout-vent length (cm)	Methyl ketone expression ($\mu g/cm^2$)	Unique methyl ketones observed	Relative (%) methyl ketone concentrations:	
				Saturated	Unsaturated
1	48.0	5.6	17	75.1	24.9
2	50.4	2.5	12	74.8	25.2
3	54.0	2.2	17	70.3	29.7
4	55.5	2.3	18	58.5	41.5
5	55.6	4.3	18	53.0	47.0
6	56.0	2.1	15	55.9	44.1
7	57.5	16.4	18	49.1	51.9
8	58.5	2.0	16	70.8	29.2
9	59.2	7.0	17	71.5	28.5
10	59.8	1.8	10	17.5	82.5
11	60.4	1.9	15	57.4	42.6
12	61.3	3.4	14	11.1	88.9
13	61.8	3.4	15	60.3	39.7
14	62.0	3.6	18	54.1	45.9
15	66.6	5.4	14	26.3	73.7
16	68.5	5.4	13	13.7	86.3
17	69.1	4.9	10	11.6	88.4
18	69.6	2.5	13	7.4	92.6
19	71.5	10.2	15	20.4	79.6
20	74.2	5.3	16	25.2	74.8

as long-chain saturated methyl ketones while the remaining nine had mass spectra in accord with long-chain ω-9 cis-saturated methyl ketones (Mason et al. 1990). The saturated methyl ketones ranged in size from 394 mass units to 506 mass units whereas the unsaturated methyl ketones ranged in size from 420 mass units to 532 mass units.

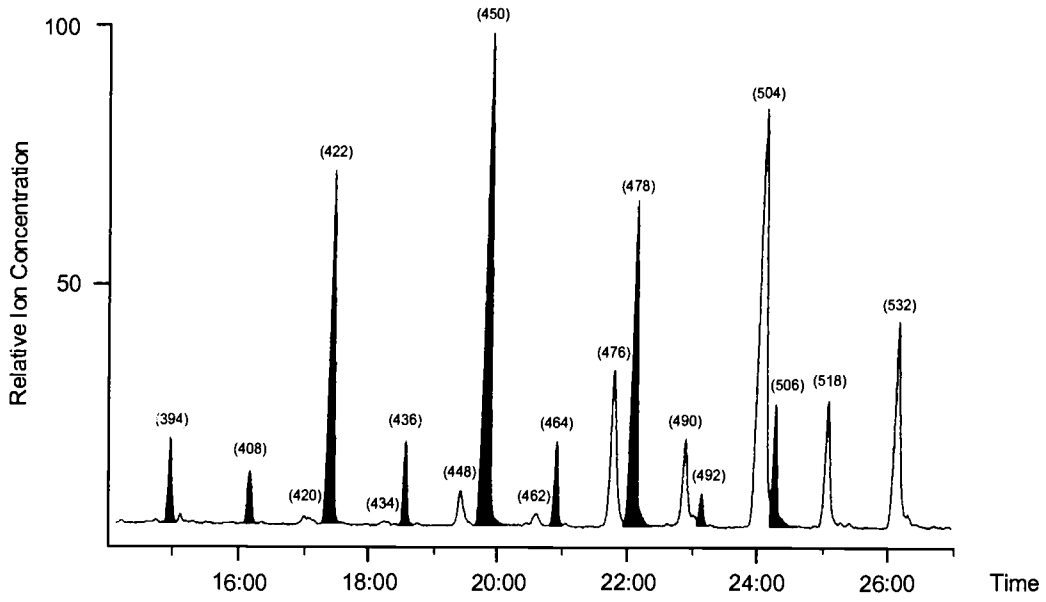
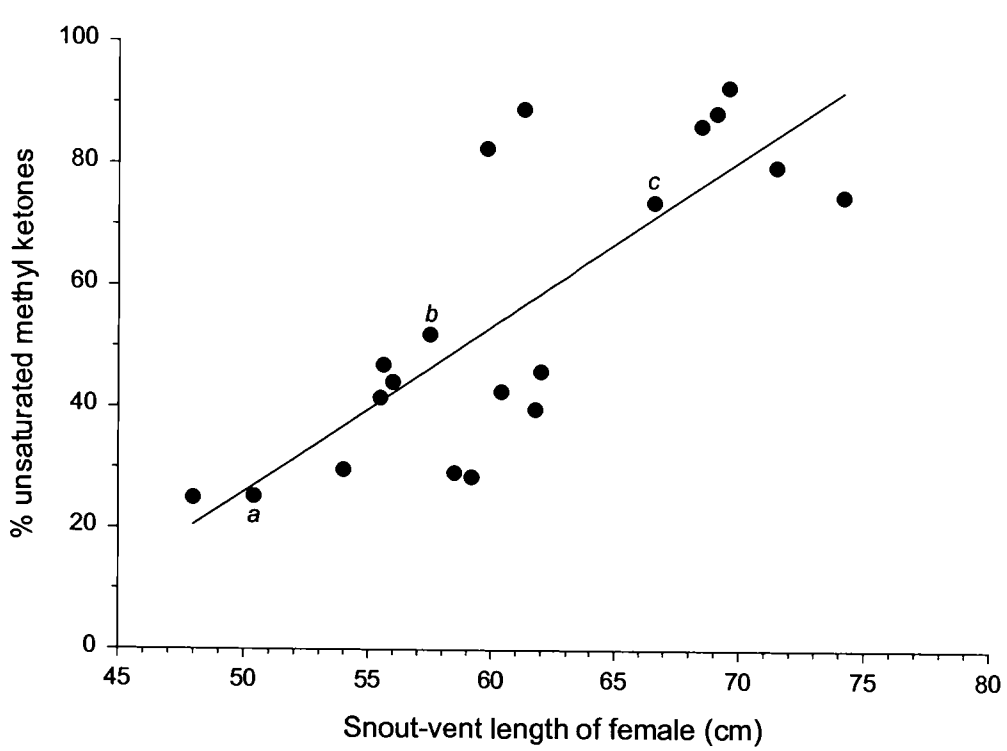


Figure 5.3. Gas chromatogram of the female sexual attractiveness pheromone profile for the red-sided garter snake, *Thamnophis sirtalis parietalis*. Pheromone profiles are composed of saturated (shaded peaks) and unsaturated (open peaks) methyl ketones. The numbers over the peaks represent the molecular weights of individual components.

Individual females were found to vary in the number of methyl ketones expressed in their skin lipids, ranging from pheromone profiles composed of 10 unique methyl ketones to pheromone profiles composed of all 18 unique methyl ketones (Table 5.2). However, when we plotted the number of unique methyl ketones present against the snout-vent length of the females, a significant relationship was not observed (Pearson product-moment correlation: $r = -0.338$, $N = 20$, $P = 0.144$).

The relative contribution of the saturated and unsaturated methyl ketones to the overall pheromone profiles varied extensively among females (Table 5.2). For example, one female expressed a pheromone profile composed of 75.1 % saturated methyl ketones and 24.9 % unsaturated methyl ketones whereas a second female expressed a profile

composed of 7.4 % saturated methyl ketones and 92.6 % unsaturated methyl ketones. On average (± SE), saturated methyl ketones composed 44.2 (± 5.5) % of the total methyl ketones in the pheromone profiles and unsaturated methyl ketones composed 55.8 (± 5.4) %. When we plotted the snout-vent length of the females against the relative contribution of unsaturated methyl ketones to the pheromone profiles, a significant correlation was observed (Pearson product moment correlation: $r = 0.788$, $N = 20$, $P < 0.001$; Figure 5.4). This relationship showed that smaller females express pheromone profiles composed predominately of saturated methyl ketones while larger females expressed profiles composed predominately of unsaturated methyl ketones.



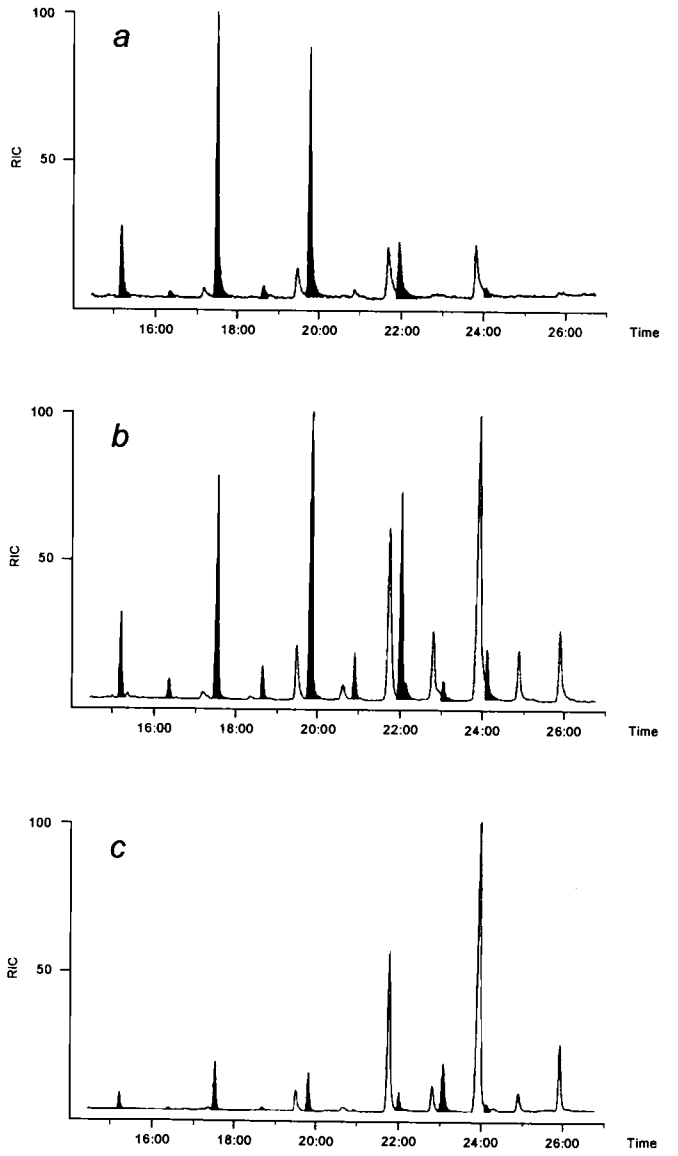


Figure 5.4. Regression of the percent relative concentration of unsaturated methyl ketones composing the sexual attractiveness pheromone profile on snout-vent length for female red-sided garter snakes, *Thamnophis sirtalis parietalis*. Small females express pheromone profiles consisting predominately of saturated methyl ketones (a), mid-size females express profiles consisting of equal proportions of saturated and unsaturated methyl ketones (b), and large females express profiles consisting predominately of unsaturated methyl ketones (c).

Discussion

The results of this study demonstrate that adult red-sided garter snakes are able to discriminate between females of various size based solely on chemical cues contained with the skin lipids of the female. Males displayed a courtship preference for large females over small females when presented with both simultaneously and in isolation and continued to demonstrate a similar preference when visual, tactile, and behavioral cues from the females were removed through the use of skin lipid extracts. Furthermore, we observed size-specific variation in the chemical structure of the female sexual attractiveness pheromone, strongly suggesting that this pheromone represents the skin lipid-borne cue responsible for mediating male mate choice in this species. Previous studies have documented chemically mediated mate choice in a variety of vertebrates (*e.g.*, mammals: lemmings – Huck & Banks, 1982 and rabbits – Reece-Engel, 1988; amphibians: salamanders – Verrell, 1985, Marco *et al.*, 1998; reptiles: lizards – Martín & López, 2000), but have focused exclusively on the behavioral responses of individuals to chemical cues. To our knowledge, this study represents the first attempt to provide empirical evidence towards the identification of a chemical cue responsible for mediating mate choice in a vertebrate species.

Our results from the behavioral experiments confirm previous studies documenting a courtship preference by male red-sided garter snakes for larger females during the breeding season (Hawley & Aleksiuk, 1976; Garstka *et al.*, 1982; Shine *et al.*, 2001). Similar courtship preference by males for larger females has been observed in other reptiles including lizards (*e.g.*, Olsson, 1993; Cooper & Vitt, 1997; Whiting & Batemen, 1999) and snakes (*e.g.*, Luiselli, 1996). One potential benefit incurred by male

red-sided garter snakes for choosing a female of larger body size is an increase in the number of offspring sired. Fecundity has been shown to increase with body size in a variety of animals (reviewed in Andersson, 1994), and the same appears true for red-sided garter snake populations in Manitoba where larger females produce larger clutches of young (Gregory, 1977; R.T. Mason, unpublished data).

We are confident that our bioassay results represent a true measure of male courtship preference for larger female skin lipids. When male red-sided garter snakes were presented with skin lipid extracts from both large and small females, we observed courtship behavior closely mimicking what was observed with the actual females, including chin-rubbing and body alignment (with edge of filter paper). These behaviors are only observed in a reproductive context (Mason, 1993) confirming that we were measuring a courtship response and not some other male behavior (*e.g.*, aggregation). Furthermore, analysis of the treated filter papers showed that similar amounts of skin lipids were transferred to the filter papers from the large and small snakes. Potentially, variation in the total amount of skin lipids transferred for the two female sizes could have driven the male courtship preference observed. Instead, our results suggest that there is some intrinsic property of the female skin lipids that allows for males to differentiate among large and small females.

The observed relationship between the snout-vent length of females and the methyl ketone group ratios demonstrates that the sexual attractiveness pheromone can function as a reliable indicator to males of female body size. Indeed, the observed uniform shift in methyl ketone group ratios as females increased in size conforms well with the observed uniform change in male courtship behavior displayed to females of

varying size when tested in the field (Hawley & Aleksiuk, 1976). In addition, the pattern of variation we observed in pheromone composition is concordant with what would be expected for such variation to function in male mate selection. Although both saturated and unsaturated methyl ketones are required to elicit full male courtship behavior in male red-sided garter snakes, unsaturated methyl ketones appear to be the more biologically active of the two groups. When presented in isolation, unsaturated methyl ketones elicit a five-fold increase in male response over saturated methyl ketones (Mason *et al.*, 1989). Accordingly, higher levels of unsaturated methyl ketones in the pheromone ratios of larger females, as were observed in this study, is a likely explanation for why males find these females more attractive.

Why larger females express higher levels of unsaturated methyl ketones is unclear. The production and expression of this pheromone appears to be under hormonal control (estrogen – Crews, 1976, 1984), suggesting that variation in the release rate of hormones and/or sensitivity of target cells to the hormones may explain the variation. For example, larger females produce greater number of follicles in the fall (hence, greater numbers of offspring – Gregory, 1977) which may translate into higher levels of estrogen circulating in the blood during the period of pheromone biosynthesis. Alternatively, females of varying size may differ in the organization of biosynthetic pathways involved in methyl ketone production. Future studies are necessary to resolve the underlying mechanism(s) responsible for the observed variation in methyl ketone production.

Potentially, we might have expected to find variation in the quantity of sexual attractiveness pheromone expressed by female red-sided garter snakes of varying size or variation in the presence or absences of individual methyl ketones. Similar mechanisms

of mate choice have been observed in insects (reviewed in Andersson, 1994). For example, female rattlebox moths (*Utetheisa ornatrix*) prefer to mate with males producing higher quantities of a courtship pheromone (Dussourd *et al.*, 1991) whereas female oriental moths (*Grapholita molesta*) preferentially court males expressing a food-related chemical in their pheromone blend (Lofstedt *et al.*, 1989). However, we did not observe a correlation between female body size and either of the two measures in the expression of the sexual attractiveness pheromone suggesting that such variation is not utilized by male red-sided garter snakes to differentiate among females.

Although our results from this study strongly suggest that male red-sided garter snakes differentiate among females of varying size utilizing size-specific variation in the female sexual attractiveness pheromone, we can not rule out additional cues which may assist males in mate choice for this species (Shine & Mason, 2001). For example, male garter snakes searching for sexually attractive females in the field are initially attracted to movement (Joy & Crews, 1988; Holtzman, 2001). Thus, males might initially respond to perceived areas of greater movement (i.e. larger female moving through grass or action of larger mating ball) and then confirm their choice by sampling the skin lipid composition of the female once contact is made. In addition, tactile cues may provide additional relevant information to the male. When a male initially comes in contact with a female, he proceeds to chin-rub up and down the female turning back at the head and tail before finally coming to rest with his head resting behind the female's head (Noble, 1937). Males perform this behavior to detect quickly whether the female is mated by means of post-copulatory pheromones deposited near the cloaca (Shine *et al.*, 2000), but males

might also be able to judge the size of the female based on distance covered. Further studies are required to determine to what extent these additional cues may be utilized.

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Chapter 6

Pheromonally-mediated Sexual Isolation among Denning Populations of Red-sided Garter Snakes, *Thamnophis sirtalis parietalis*

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To be submitted to *Journal of Chemical Ecology*,
Plenum Press, New York, New York
33 pages

Abstract

Utilizing behavioral experiments and chemical analyses, we examined whether pheromonally-mediated sexual isolation exists between denning populations of red-sided garter snakes (*Thamnophis sirtalis parietalis*) in Manitoba, Canada. Simultaneous choice tests conducted during the breeding season revealed that adult males from a hibernaculum in central Manitoba displayed a strong courtship preference for females from their own population over females from a hibernaculum in western Manitoba, whereas males from the western Manitoba hibernaculum showed no such preference. In addition, trailing experiments testing the response of males from the two hibernacula to familiar and unfamiliar female trails showed similar results, demonstrating that the observed male preference is mediated through chemical cues. Subsequent chemical analysis of the female sexual attractiveness pheromone, a homologous series of long-chain saturated and ω-9 cis-unsaturated methyl ketones responsible for eliciting male courtship behavior and trailing behavior in garter snakes, showed significant variation in the composition of the pheromone between the two populations. Specifically, the two populations varied in the relative concentrations of individual unsaturated methyl ketones expressed by females. These results suggest that sexual isolation exists to a degree among denning populations of red-sided garter snakes due to variation in the expression of the female sexual attractiveness pheromone.

Introduction

Studies utilizing insect models have dominated pheromone research in recent decades, resulting in the chemical identification and characterization of over a thousand

pheromones (Abelson, 1985). Knowledge of the chemical structure of insect pheromones has allowed investigators to make significant advances in the understanding of how these chemical cues regulate insect behavior (Bell & Cardé, 1984; Eisner & Meinwald, 1995). In contrast to the abundance of pheromones identified in insects, very few vertebrate pheromones have been isolated and identified. These include pheromones for fish (*e.g.*, goldfish – Stacey & Sorenson, 1986), amphibians (*e.g.*, newts – Kikuyama *et al.*, 1995; salamanders – Rollmann *et al.*, 1999; frogs – Wabnitz *et al.*, 1999), reptiles (*e.g.*, snakes – Mason *et al.*, 1989) and mammals (*e.g.*, pig – Patterson, 1968; elephant – Rassmussen *et al.*, 1996).

The lack of knowledge regarding the basic structure of vertebrate pheromones has hampered our efforts to understand vertebrate pheromone systems and has left many basic areas of research unexplored. For example, few studies have attempted to address intraspecific variation in the chemical composition of a vertebrate pheromone at the level of the population (*e.g.*, Rollman *et al.*, 2000). In insects, intraspecific variation in sex pheromone expression among geographically isolated populations is commonly observed (*e.g.*, turnip moth – Löfstedt *et al.*, 1986, Wu *et al.*, 1999; corn borer – Huang *et al.*, 1998). Such variation often results in the disruption of courtship and mating between the populations involved (*e.g.*, pine beetle –Lanier *et al.*, 1972, Miller *et al.*, 1997). Similar occurrences of pheromonally mediated sexual isolation may exist within vertebrate species, but this has yet to be explored.

The red-sided garter snake (*Thamnophis sirtalis parietalis*) is the most northern living reptile in the Western Hemisphere (Logier & Toner, 1961). Annual aggregations of red-sided garter snakes at underground hibernacula in Manitoba, Canada, are unique

natural phenomena representing the highest concentration of snakes in the world (Gregory, 1984). Marshes, shallow lakes, and areas of poor drainage offer good summer feeding grounds for the snakes while limestone bedrock provides hibernaculum sites where the snakes are constrained to spend up to eight months of the year to avoid the harsh winters (Aleksiuk & Stewart, 1971). A scarcity of suitable hibernation sites, coupled with a high degree of den fidelity and mating occurring almost exclusively at the dens (Gregory, 1974, 1977), has effectively divided the red-sided garter snakes in this region into geographically isolated populations defined by particular hibernacula.

Mating of the red-sided garter snake occurs directly at the hibernaculum following spring emergence (Gregory, 1974). Like most snakes (reviewed in Mason, 1992), the regulation of reproductive behaviors in the red-sided garter snake depends primarily on the production and expression of specific sex pheromones (Mason, 1993). Of particular importance is the sexual attractiveness pheromone. Composed of a homologous series of saturated and ω-9 cis-unsaturated methyl ketones (Mason *et al.*, 1989; Mason *et al.*, 1990), this pheromone is sequestered in the skin lipids of females and is responsible for eliciting male courtship behavior (Noble, 1937; Garstka *et al.*, 1982). If a male does not detect the sexual attractiveness pheromone then courtship will not be initiated and subsequent mating will not occur (Mason, 1993).

The reliance of the red-sided garter snake on the sexual attractiveness pheromone to initiate reproductive behavior, coupled with its discontinuous population structure in Manitoba, offers a strong basis for investigating pheromonally-mediated sexual isolation in this species. Here we present a study initially designed to test the courtship preference of male red-sided garter snakes from two geographically isolated hibernacula to females

from their own den versus females from the distant den. Because a courtship preference was detected for males from one of the dens, we then set out to determine whether the observed preference was mediated through variation in the sexual attractiveness pheromone. This was accomplished by 1) utilizing trailing experiments to determine whether the observed preference was mediated through chemical cues, and 2) performing chemical analysis to evaluate whether detectable variation existed in the female sexual attractiveness pheromone between the two hibernacula.

Materials and Methods

Study Populations

Red-sided garter snakes used in this study were obtained from two hibernacula located approximately 240 km apart in Manitoba, Canada (Figure 6.1). The Narcisse den, located in the Interlake region of Manitoba (50°44.37N, 97°31.26W), houses in excess of 20,000 snakes during the winter months, whereas the Reeder den, located in western Manitoba (50°06.34N, 101°06.32W), shelters approximately 10,000 individuals (R.T. Mason, unpublished data). Snakes from both populations demonstrate similar temporal patterns; individuals emerge in late April, participate in a brief mating season at the hibernaculum, and then migrate to the summer feeding grounds before returning to the hibernacula in early September (Gregory, 1977). Adult females from the two populations attain an average snout-vent length (SVL) of 55-60 cm and a mean mass of 70-75 g. Adult males are smaller, attaining an average SVL of 45-50 cm and a mean mass of 35-40 g in the Narcisse population and an average SVL of 50-55 cm and a mean

mass of 42-47 g in the Reeder population (Shine *et al.*, 1999; M.P. LeMaster, unpublished data).

Behavioral Experiments

Experimental Animals

Unmated female red-sided garter snakes (n = 11 per hibernaculum) and sexually active adult male snakes (n = 150 per hibernaculum) were collected at the two hibernacula immediately upon emergence in May of 1998. To avoid any confounding effects due to temporal variation in mating behavior during the breeding season, snakes

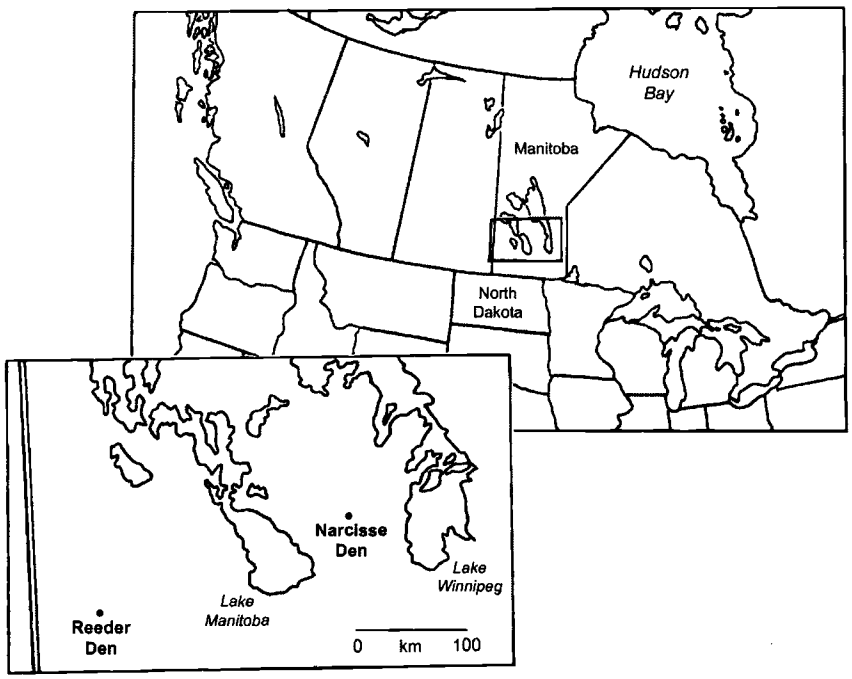

Figure 6.1. Map of Manitoba, Canada showing the location of the two populations of red-sided garter snakes used in this study. The Narcisse den population over-winters in a limestone sinkhole, whereas the Reeder den population shelters in a shale outcrop.

were collected from the two hibernacula over a single 24-hour period. Animals were segregated by sex and hibernaculum in cloth bags and returned to the Chatfield Research Station (Chatfield, Manitoba) where they were held at ambient temperature until testing. Following testing, the animals were returned to their respective hibernacula and released.

Courtship Trials

To examine whether red-sided garter snakes preferentially court females from their own hibernaculum, we tested the courtship response of males from the Narcisse and Reeder den to resident (same den) and non-resident (distant den) females utilizing a simultaneous choice test design (Mason & Crews, 1985). Briefly, we introduced ten randomly chosen males from a particular den into an outdoor arena (1 x 1 x 1 m; constructed of nylon cloth – Moore *et al.*, 2000) and allowed them to acclimate for five minutes. We then placed into each arena simultaneously a female from the Narcisse den and a female from the Reeder den. The two females were matched according to snout-vent length, as male red-sided garter snakes demonstrate a strong courtship preference for females of larger size (Shine *et al.*, 2001). In addition, tape was placed across the cloaca of each female to prevent females from mating during the trials, an event that drastically reduces further male courtship behavior (Garstka *et al.*, 1982). Following the introduction of the females, the snakes were allowed to interact undisturbed for five minutes after which time the number of males actively courting each female was recorded.

Table 6.1. Ethogram of courtship behavior for the male red-sided garter snake, *Thamnophis sirtalis parietalis* (Modified from Crews *et al.*, 1984). A behavioral score of 2.0 or greater only occurs in a reproductive context and is therefore indicative of sexual behavior for males of this species.

Courtship Score	Description of Behavior
1.0	Male investigates female with increasing tongue-flick rate
2.0	Male chin-rubs dorsal surface of female and aligns body with female
3.0	Male actively tail searches and attempts cloacal apposition with female
4.0	Male copulates with female

We performed two experiments testing the courtship response of Narcisse den males (n = 9 trials) and Reeder den males (n = 9 trials) to resident and non-resident females. For each experiment, test males were replaced with new males following each trial. In addition, female pairs of similar size were only used once per experiment. Male courtship behavior was assessed using an ethogram of male garter snake mating behavior (Table 6.1). For a male to be considered actively displaying courtship behavior during the trials, a score of 2.0 or greater was required on the ethogram. To test for significant male responses in the two experiments, the difference in the number of simultaneous choice tests ending with more males courting females from their own hibernaculum verses females from the distant hibernaculum were analyzed using Wilcoxon sign-rank tests (Sokal & Rohlf, 1990). Level of significance for each test was set at $P < 0.05$.

Trailing Experiments

To determine whether the male red-sided garter snake courtship preference observed is mediated through skin-derived chemical cues, we tested the trailing response of male red-sided garter snakes when presented with resident and non-resident females on

a standard Y-maze previously described by LeMaster and Mason (2001). We performed two experiments testing the trailing preference of Narcisse den males (n = 11 trials) and Reeder den males (n = 11 trials). We also performed a control experiment (n = 10 trials) at the onset of the study to verify that there was no bias in arm choice by males when no stimuli were present (both arms of the maze blank). In each experiment, unique males were used for each trial. In addition, female pairs (the same nine pairs used in the courtship experiments plus additional two pairs) were used only once per experiment. Trials were conducted daily between the hours of 1000 to 1600, when the snakes were normally active in the field (M.P. LeMaster, personal observation) and environmental conditions (*e.g.,* temperature, lighting) remained constant throughout the experimental period. Statistical significance in male trailing behavior was analyzed utilizing one-way binomial tests (Sokal & Rohlf, 1990). Level of significance for each test was set at $P < 0.05$.

For each trial, we randomly selected a pair of stimulus females and rubbed the dorsal surface of each female on the surface of the maze. Trails were placed side-by-side on the base arm and then crossed over each other at the Y-junction before exiting out separate, randomly chosen arms. The cloacal openings of the donor females were covered during transfer of the skin lipids so that contamination from cloacal gland secretions did not occur. We then placed a male test snake (Narcisse or Reeder den male depending on experiment) into an opaque box at the start of the base arm and allowed it to enter the maze of its own accord. Trials ended when the test snake completely entered one exit arm of the Y-maze. Trials in which the test snake failed to exit the hide box after ten minutes or trials in which the test snake fell from the maze were eliminated (< 4% of

trials performed). For all successful trials, we recorded the arm chosen by the test snake. We also noted whether the test snake displayed a trail contact response (TCR), an overt response characterized by a snake placing its chin in contact with the substrate and investigating with short, rapid tongue-flicks followed by subsequent movement along the pre-existing trail (Brown & MacLean, 1983).

Chemical Analysis

Pheromone Collection

Adult, sexually attractive female red-sided garter snakes from the Narcisse den (n = 10) and the Reeder den (n = 12) were collected immediately upon spring emergence from the hibernaculum in May of 1999. We focused our collection on females in a particular size range (SVL = 62.0 – 75.0 cm) as the sexual attractiveness pheromone of female red-sided garter snakes varies qualitatively in relation to female body length (LeMaster, unpublished data). The animals were killed with an overdose of brevital sodium and each snake was then placed dorsal side down in a 500-ml glass beaker and covered with 25-50 ml of 100% hexane (C_6H_{12}) (Mason *et al.*, 1989; Mason *et al.*, 1990). Following a 12-hour immersion period, the animals were removed from the hexane, and the excess solvent was removed under reduced pressure by rotoevaporation at 35°C. The resulting residues were weighed on a digital scale (Mettler AT400), resuspended in fresh hexane (1-2 ml), and sealed in 9-ml glass vials with polyethylene-lined caps for storage at -20°C.

To isolate the methyl ketones composing the sexual attractiveness pheromone, we fractionated the skin lipid extracts using column chromatography as described by Mason *et al.* (1989). Briefly, we loaded the skin lipid extracts onto glass columns (350 mm x 22 mm ID) packed with alumina (activity III) and eluted the columns with hexane and ethyl ether ($C_4H_{10}O$) solutions of increasing polarity. For each sample, the fraction containing the appropriate methyl ketones (fraction 5 – Mason *et al.*, 1989) was collected and the excess solvent was removed by rotoevaporation (35°C). The resulting methyl ketone residues were weighed on a digital scale (Mettler AT400) and resuspended in fresh hexane (1 ml). Samples were then placed in 9-ml glass vials with polyethylene-lined caps and stored at –20°C until further analysis.

Pheromone Analysis

Multi-component pheromones, such as the sexual attractiveness pheromone of the red-sided garter snake, can show variation in both the quantity and quality of pheromone expressed. To examine variation in the quantity of pheromone expressed by female snakes from the two hibernacula, we calculated the amount of methyl ketones expressed per unit skin surface area ($\mu g/cm^2$) for individual females. This was accomplished by dividing the weight of the isolated methyl ketone residues extracted from a female by the total skin surface area of the female. A general measure of skin surface area for each female was determined by multiplying the snout-vent length of a female by its circumference at mid-body (Mason *et al.*, 1990).

To examine variation in the quality of pheromone expressed by females from the two populations, we determined the number of unique methyl ketones expressed by

individual females and compared the relative concentrations of individual methyl ketones comprising the overall pheromone profiles for the two populations. The methyl ketones present in the pheromone extracts were identified utilizing a Hewlett Packard 5890 Series II gas chromatograph fitted with a split injector (280°C) and a Hewlett Packard 5971 Series mass selective detector. Aliquots (1 µl) of the methyl ketone fractions were injected onto a fused-silica capillary column (HP-1; 12 m x 0.22 mm ID; Hewlett Packard, California, USA) with helium as the carrier gas (5 cm/sec). Oven temperature was initially held at 70°C for 1 min, increased to 210°C at 30°C/min, held at 210°C for 1 min, increased to 310°C at 5°C/min, and finally held at 310°C for 5 min. Once the methyl ketones were identified, we then calculated the relative concentrations of individual methyl ketones in each sample using peak integration. Identification of compounds and peak areas were determined utilizing ChemStation software (Version B.02.05; Hewlett Packard, California, USA) interfaced with the gas chromatograph/mass spectrometer.

Statistical significance in the amount of sexual attractiveness pheromone expressed on the dorsal surface of the female and the total number of unique methyl ketones expressed were examined using a Student t-test and a Mann-Whitney rank sum test, respectively (Sokal & Rohlf, 1990). We used a randomization test (Manly, 1991) to test for a difference in the relative concentrations of individual methyl ketones composing the sexual attractiveness pheromone among the two dens. The test statistic was the Euclidean distance (S) between the average proportions of all individual methyl ketones observed. The randomization test generated a p-value by comparing the observed test

statistic to a simulated distribution based on 10,000 reshufflings of the data set. Level of significance for all tests was set at $P < 0.05$.

Results

Behavioral Experiments

Courtship Experiments

The average SVL (± SD) of Narcisse females used in the simultaneous choice tests was 59.0 (± 8.9) cm whereas the average SVL (± SD) for Reeder females was 60.1 (± 10.0) cm. When paired for individual trials, the average SVL difference (± SD) among females from the two hibernacula was 1.5 (± 1.2) cm. Narcisse den females were observed to be slightly larger in four of the nine trials for each experiment while Reeder den females were slightly larger in the remaining five trials.

When presented with the females from the two hibernacula, male garter snakes responded with stereotypical courtship behaviors including increased tongue-flick rate and chin rubbing along the dorsum of the females. Narcisse den males displayed a significant courtship preference for females from their own den over females from the Reeder den; in seven of nine trials conducted, a greater proportion of the males were observed courting the Narcisse den females (Wilcoxon signed-rank test: $W = 33$, $N = 9$, $P = 0.016$; Figure 6.2a). On average across the trials, 43.3 % of the males were found courting the Narcisse females whereas only 17.8 % of the males were observed actively courting the Reeder females. Interestingly, Reeder den males did not demonstrate a

similar courtship preference to the Narcisse den males; in only four of the nine trials conducted was a greater proportion of the Reeder den males observed courting the Reeder den female (Wilcoxon signed-rank test: $W = 2$, $N = 9$, $P = 0.945$; Figure 6.2b). Overall, 25.6 % of the males were found courting the Narcisse females whereas 26.7 % of the males were observed courting the Reeder females.

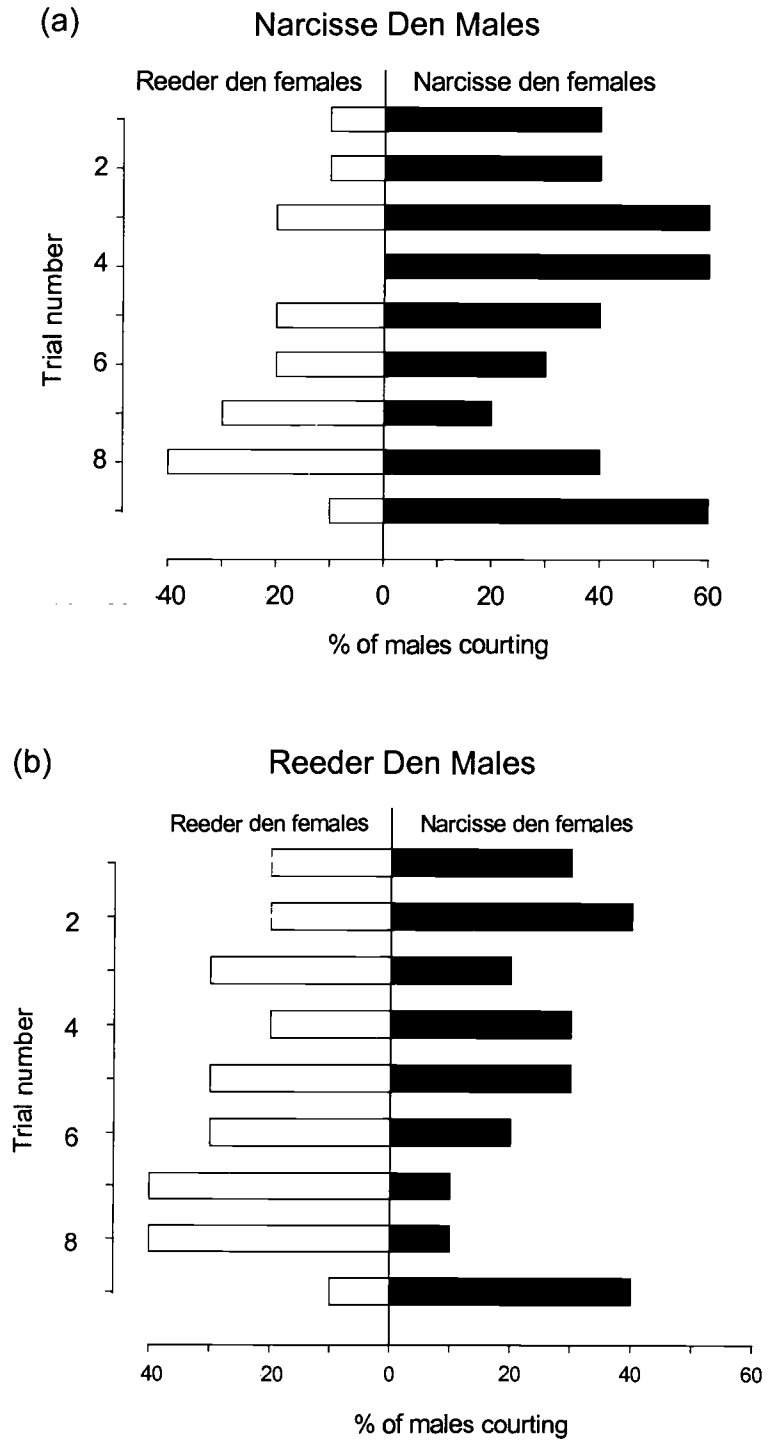

Figure 6.2. Results of simultaneous choice tests in which a familiar and an unfamiliar female red-sided garter snake (*Thamnophis sirtalis parietalis*) were placed in an outdoor arena with 10 courting males from (a) Narcisse den and (b) Reeder den. After five minutes the percentage of males courting each female was recorded.

Table 6.2. Trailing response of male red-sided garter snakes (*Thamnophis sirtalis parietalis*) from two separate hibernacula to females from their own den versus females from a distant den when presented simultaneously on a Y-maze.

Condition	N	Arm Treatment	Number of Times Selected	P
Control	10	Blank	6	0.205
		Blank	4	
Narcisse males tested on Narcisse *versus* Reeder female trails	11	Narcisse female	9	0.027
		Reeder female	2	
Reeder males tested on Narcisse *versus* Reeder female trails	11	Narcisse female	5	0.226
		Reeder female	6	

Trailing Experiments

When presented with the control maze (both arms blank), male red-sided garter snakes showed no preference for one arm over the other (binomial test; P = 0.205; Table 6.2). When tested with the simultaneous trails, males in both experiments responded with trail contact responses upon first exiting the start box, regardless of which trail a male might have initially encountered. Overall, Narcisse den males displayed a strong trailing preference for females from their own hibernaculum over females from the Reeder hibernaculum, choosing the arm with the Narcisse den female trail in 9 of the 11 trials conducted (binomial test; P = 0.027; Table 6.2). Reeder den males, however, showed no such preference among the trail types, appearing to randomly chose among the trails at the Y-junction (binomial test; P = 0.226; Table 6.2).

Pheromone Analysis

Pheromone Quantity

There was no difference in the average snout-vent length (Narcisse den females = 68.3 (± 4.9) cm; Reeder den females = 69.6 ± (6.6) cm: Student t-test; t = -0.522, P = 0.607) or average mass (Narcisse den females = 114.7 (± 31.0) g; Reeder den females = 106.2 (± 28.7) g: Student t-test; t = -0.664, P = 0.514) among the females sampled for pheromone analysis. The hexane extractions of individual females yielded an average (± SE) of 25.1 (± 2.8) mg of skin lipids per female from the Narcisse den and 22.8 (± 3.5) mg of skin lipids per female from the Reeder den. Subsequent fractionation of the lipids yielded an average (± SE) methyl ketone fraction of 4.8 (± 0.9) mg per Narcisse den female and 4.2 (± 0.7) mg per Reeder den female. Overall, the methyl ketones accounted for an average (± SD) of 20.0 (± 10.6) % of the skin lipids collected from the Narcisse den females and 19.5 (± 7.0) % of the skin lipids collected from the Reeder den females. After accounting for variation in skin surface area, we did not observe a significant relationship between the females from the two hibernacula and the amount of methyl ketones extracted per unit of surface area (Student t-test; t = 0.561, P = 0.581; Table 6.3).

Table 6.3. Quantitative and qualitative variation in expression of the sexual attractiveness pheromone among female red-sided garter snakes (*Thamnophis sirtalis parietalis*) from two denning populations in Manitoba, Canada.

Female	Snout-vent length (cm)		Mass (g)		Methyl ketone expression ($\mu g/cm^2$)		Unique methyl ketones expressed	
	Narcisse den	Reeder den	Narcisse den	Reeder den	Narcisse den	Reeder den	Narcisse den	Reeder den
1	74.2	63.0	116.5	80.1	5.3	7.0	16	18
2	62.0	77.8	75.5	144.3	3.6	20.4	18	17
3	71.5	62.5	125.5	74.5	10.2	7.3	16	18
4	63.3	72.6	95.9	124.4	6.3	23.0	17	18
5	61.9	69.5	90.9	101.9	13.2	8.8	17	17
6	73.2	62.7	129.2	75.4	22.4	10.9	17	17
7	73.9	75.2	190.0	134.7	9.6	12.0	17	18
8	70.4	72.6	102.6	113.3	14.5	8.1	17	17
9	66.7	80.7	110.7	136.8	17.0	6.9	17	18
10	66.0	62.3	110.1	73.9	28.6	11.0	17	17
11	---	72.5	---	137.6	---	12.0	---	17
12	---	64.1	---	77.8	---	10.6	---	17
Average: (± SD)	68.3 (± 4.9)	69.6 (± 6.6)	114.7 (± 31.0)	106.2 (± 28.7)	13.1 (± 7.9)	11.5 (± 5.1)	16.9 (± 0.57)	17.4 (± 0.52)

Pheromone Quality

Complete GC-MS analysis of the methyl ketone fractions revealed the presence of eighteen unique long-chained methyl ketones (Figure 6.3). Nine of these were identified as long-chain saturated methyl ketones while the remaining nine had mass spectra in accord with long-chain ω-9 cis-unsaturated methyl ketones (Mason *et al.*, 1990). The saturated methyl ketones ranged in size from 394 mass units to 506 mass units whereas the unsaturated methyl ketones ranged in size from 420 mass units to 532 mass units. Females from the two hibernacula were found to vary in the number of methyl ketones expressed in their skin lipids, ranging from pheromone profiles composed of 16 unique methyl ketones to pheromone profiles composed of all 18 unique methyl ketones.

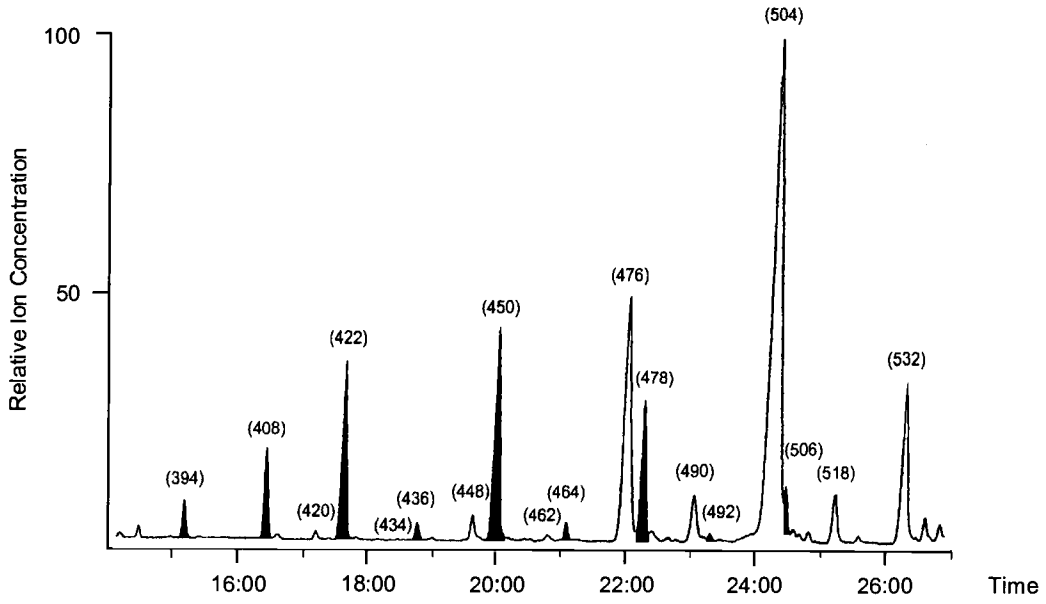

Figure 6.3. Gas chromatogram of the female sexual attractiveness pheromone profile for the red-sided garter snake, *Thamnophis sirtalis parietalis*. Pheromone profiles are composed of saturated (shaded peaks) and unsaturated (open peaks) methyl ketones.

However, when we compared the average number of methyl ketones expressed by females from each hibernacula, a significant relationship was not observed (Mann-Whitney rank sum test; T = 92.5, P = 0.144; Table 6.3).

Although the two hibernacula did not vary significantly in the number of unique methyl ketones expressed, we did observe significant variation in the relative contribution of the individual methyl ketones composing the pheromone profiles for the two hibernacula (randomization test; S observed = 0.14, S generated = 0.07, P = 0.038; Figure 6.4a). Subsequent analyses showed that this difference was not due to variation between hibernacula in the relative contribution of total saturated versus unsaturated methyl ketones (t-test; t = 1.492, P = 0.151). Instead, independent analyses of the two methyl ketone groups revealed that the two hibernacula varied significantly in the relative concentration of individual unsaturated methyl ketones (randomization test; S observed = 0.14, S generated = 0.06, P = 0.011; Figure 6.4b) whereas the relative concentration of saturated methyl ketones remained stable among dens (randomization test; S observed = 0.07, S generated = 0.05, P = 0.195; Figure 6.4c).

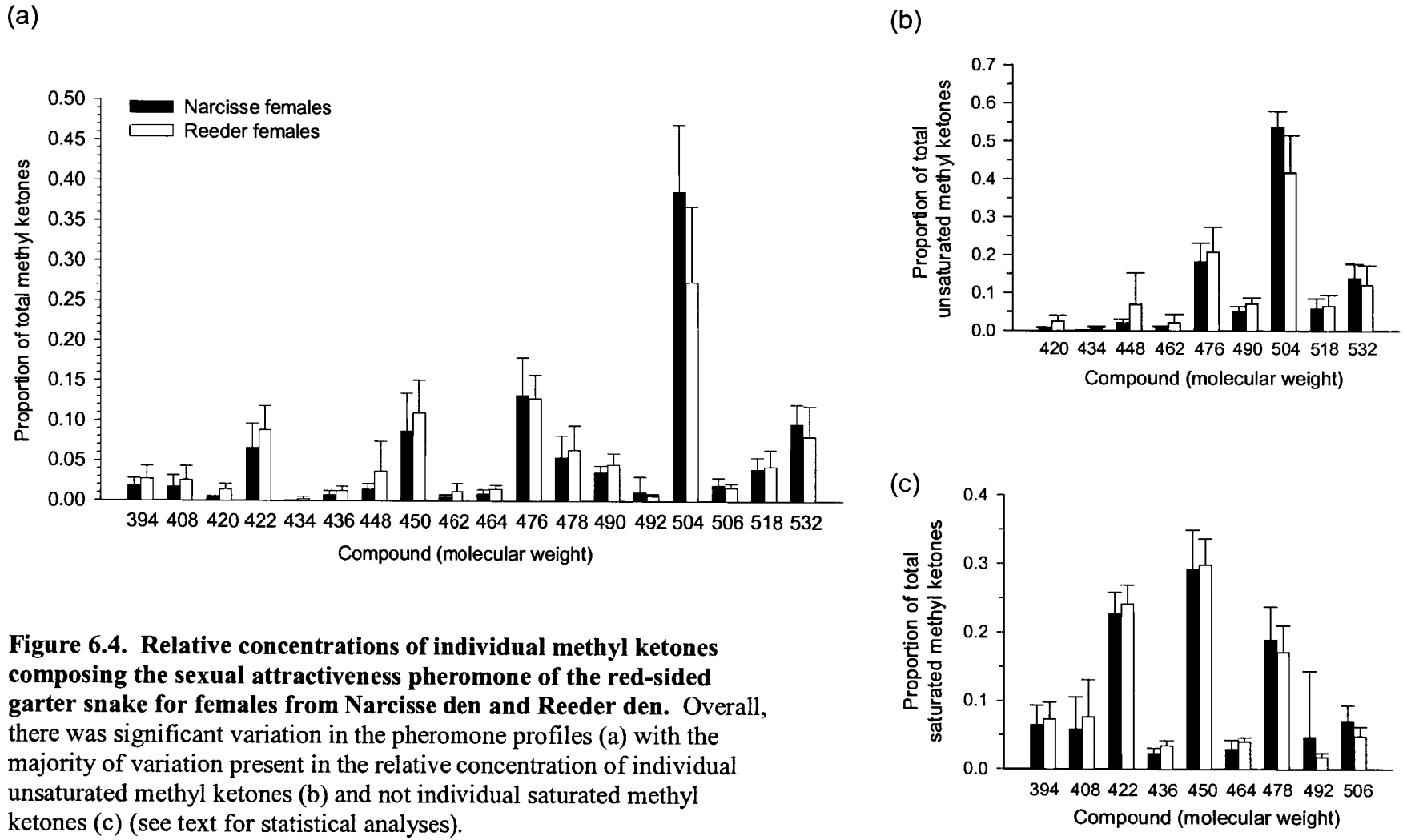

Figure 6.4. Relative concentrations of individual methyl ketones composing the sexual attractiveness pheromone of the red-sided garter snake for females from Narcisse den and Reeder den. Overall, there was significant variation in the pheromone profiles (a) with the majority of variation present in the relative concentration of individual unsaturated methyl ketones (b) and not individual saturated methyl ketones (c) (see text for statistical analyses).

Discussion

Intraspecific sexual isolation has been documented in a variety of vertebrates including fish (*e.g.*, Ayvazian, 1993; Ziuganov, 1995), amphibians (*e.g.*, Houck *et al.*, 1988; Verrell & Arnold, 1989), and mammals (*e.g.*, Pillay *et al.*, 1995; Pillay, 2000). While multiple studies have utilized behavioral trials to explore the role of chemical cues in sexual isolation (*e.g.*, Ovaska, 1989; Pillay *et al.*, 1995), few studies have attempted to evaluate whether structural variation exists among populations for a particular chemical cue. For example, Rollman *et al.* (2000) observed biochemical variation in a male courtship pheromone among populations of salamanders of the *Plethodon jordani-glutinosus* complex, suggesting that this pheromone might be responsible for the moderate levels of sexual isolation previously observed among populations of this species. However, behavioral trials testing the receptivity of females to pheromone extracts from various populations have yet to be performed. To our knowledge, the present study represents the first attempt to integrate behavioral experiments and chemical analysis towards understanding the role of chemical cues in sexual isolation within a vertebrate species.

The results of the courtship experiments demonstrate that sexual isolation exists to a degree among denning populations of red-sided garter snakes. Males from the Narcisse den displayed a courtship preference for females from their own den over females from the Reeder den. Interestingly, males from the Reeder den did not display a similar preference indicating that male courtship preference in these two populations is asymmetrical. Similar instances of asymmetrical sexual isolation have been observed in other species (*e.g.*, Krebs, 1990; Kobayashi, 2001), including vertebrates (*e.g.*, Ayvazian,

1993; Pillay, 2000). Why these two denning populations of snakes show asymmetrical sexual isolation is not known. We suggest that this pattern might result from reproductive character displacement due to selective pressures to recognize conspecific mates; red-sided garter snakes in central Manitoba, unlike western Manitoba, share hibernacula with a second species of garter snake, the plains garter snake (*Thamnophis radix*). Thus, males in the Narcisse den may be more narrowly tuned to reproductive cues, allowing for discrimination among conspecific and heterospecific females, while the Reeder males, under no such pressure, are more broadly tuned. Alternatively, the pattern of sexual isolation observed in this study could represent a transient phenomenon, resulting from divergence in traits and mating preferences based on those traits through sexual selection acting within each population (Arnold *et al.*, 1996).

Potentially, male red-sided garter snakes may utilize multiple cues to discriminate among females from various dens. Evidence suggests that both chemical and visual cues assist males of this species in selecting among potential mates during the breeding season (Shine & Mason, 2001). However, the results of the trailing experiments demonstrate that chemical cues alone are sufficient to allow males to discriminate among resident and non-resident females. When presented with the two female trails simultaneously, we observed males to display similar preferences to those observed in the courtship trials. In addition, the similarity in male preferences observed between the courtship and trailing experiments suggests that both behaviors are mediated by a similar chemical cue. In the case of the red-sided garter snake, that cue appears to be the female sexual attractiveness pheromone (Mason *et al.*, 1989; LeMaster & Mason, 2001).

Subsequent analysis of the female sexual attractiveness pheromone from the two hibernacula demonstrated that this pheromone contains the necessary variation for it to function as a reliable indicator to males of a female's resident status. Specifically, females were found to vary between dens in the relative concentration of individual unsaturated methyl ketones composing the sexual attractiveness pheromone. We were not surprised to observe that the variation was greatest with respect to the unsaturated methyl ketones. Although both unsaturated and saturated methyl ketones are necessary to elicit full male courtship, unsaturated methyl ketones appear to be the more biologically active of the two groups, eliciting a five-fold increase in male courtship behavior over saturated methyl ketones when presented in isolation (Mason *et al.*, 1989). Thus, low levels of variation in the relative concentration of unsaturated methyl ketones between dens, as were observed in this study, are more likely to affect male courtship behavior than similar variation in saturated methyl ketones.

Why females from the two hibernacula vary in the relative concentration of individual methyl ketones is unclear. German cockroaches (*Blattella germanica*) produce a sex pheromone composed of methyl ketones (Schal *et al.*, 1990), similar to those utilized by the red-sided garter snake. In the cockroach system, the pheromone derives its origins from fatty acid biosynthesis (reviewed in Tillman *et al.*, 1999), suggesting that the sexual attractiveness pheromone of the red-sided garter snake is synthesized through a similar process. Thus, variation in the expression or activity levels of various enzymes in the biosynthetic pathway among females from various hibernacula could result in the observed variation. In addition, the fact that males from the Narcisse den, but not the Reeder den, show a courtship preference for resident over non-resident

females suggests that variation also exists among the two male detection systems. Such variation may be present at the level of the receptor neurons (*e.g.*, Wu *et al.*, 1999), located in the vomeronasal organ of the male snake (Halpern & Kubie, 1983), or may occur in higher processing centers, including the olfactory and accessory olfactory bulbs (Halpern, 1976). Future studies are necessary to resolve the underlying mechanism(s) responsible for variation of pheromone production and detection in this system.

The potential use of the female sexual attractiveness pheromone by male red-sided garter snakes to distinguish females from their own den over females from other dens suggests that this pheromone may represent a compound signal containing hierarchical mapping. Hierarchical mapping refers to signals in which differences in mean patterns are used to code for one question and different variants around that mean are then used to answer other questions (Bradbury & Vehrencamp, 1998). Thus, in the red-sided garter snake, the presence of saturated and unsaturated methyl ketones signifies the presence of a female, whereas variation in the relative concentration of individual unsaturated methyl ketones conveys the home locality of a female. Other examples of compound signals containing hierarchical mapping in vertebrates can be found in electric fish (*e.g.*, Crawford, 1992) and birds (*e.g.*, Becker, 1982).

In conclusion, the results of this study demonstrate that pheromonally mediated sexual isolation exists to a degree among denning populations of red-sided garter snakes. Male snakes from the Narcisse den displayed a courtship preference for females from their own den over females from the Reeder den and this preference was maintained when males from were presented with female trails. Further, we observed population-specific variation in the composition of the female sexual attractiveness pheromone, the

pheromone responsible for mediating courtship and trailing behavior in the red-sided garter snake, strongly suggesting that this pheromone is responsible for the observed sexual isolation in this species.

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Chapter 7 – Conclusion

Summary

The red-sided garter snake depends on the production and perception of specific sex pheromones to initiate and orchestrate reproductive behaviors. Of particular importance is the sexual attractiveness pheromone. Composed of a homologous series of saturated and monounsaturated methyl ketones, this pheromone is responsible for eliciting male courtship behaviors during the breeding season. The objective of this thesis was to determine to what extent intraspecific variation exists in the female sexual attractiveness pheromone of the red-sided garter snake and to evaluate the potential significance of this variation for this species.

The first three chapters explored seasonal variation in the expression of the sexual attractiveness pheromone and how this variation relates to male trailing behavior. In chapter two, I utilized behavioral experiments conducted in the natural environment to test the trailing response of red-sided garter snakes during the breeding and non-breeding seasons. The results showed both sexual and seasonal variability in the response of red-sided garter snakes to pheromone trails. Male garter snakes exhibited trail contact responses and followed female trails during the breeding season, while neither sex exhibited conspecific trailing behavior during the non-breeding season. These results suggest that conspecific trailing behavior is primarily utilized by red-sided garter snakes for locating potential mates during the breeding season. In addition, females appeared to avoid male trails during the non-breeding season although additional studies are

necessary to deduce whether this is a biologically significant result or an artifact of the experimental design.

In chapter three, I tested the hypothesis that male red-sided garter snake trailing behavior is mediated by the sexual attractiveness pheromone. The trailing response of male red-sided garter snakes to males, females, and she-males was examined utilizing Y-maze experiments. Unlike normal males, she-males express a methyl ketone profile very similar to that displayed by the female during the breeding season. Male snakes were observed to follow female and she-male trails when presented on the Y-maze, but did not show an overt response to male trails. In addition, males were unable to discriminate between the female and she-male trails when presented in unison. These results are concordant with the hypothesis that the sexual attractiveness pheromone mediates male reproductive trailing behavior.

The observation that trailing behavior is limited to male red-sided garter snakes during the breeding season, coupled with the evidence that the sexual attractiveness pheromone mediates this trailing behavior, predicts that variation should exist in the expression of this pheromone by females between the breeding and non-breeding seasons. In chapter four, I demonstrated that variation in the sexual attractiveness pheromone is indeed present between the two seasons. Comparison of the methyl ketone profiles showed that the profile for the breeding season was dominated by unsaturated methyl ketones, whereas the profile from the non-breeding season showed a marked decrease in the relative concentration of unsaturated methyl ketones. In addition, pheromone samples collected across three breeding season showed little variation in the relative concentrations of the methyl ketone groups, suggesting that the composition of

the female sexual attractiveness pheromone is tightly regulated across breeding seasons for this species.

The fifth chapter explored individual variation in the sexual attractiveness pheromone among female red-sided garter snakes and how this variation might relate to male mate choice. Males displayed a courtship preference for large females over small females when presented with both simultaneously and in isolation, and continued to demonstrate a similar preference when visual, tactile, and behavioral cues from the female were removed through the testing of males with skin lipid extracts. This demonstrated that chemical cues contained in the skin lipids are sufficient to allow for males to discriminate among females of varying size. Furthermore, I observed size-specific variation in the chemical structure of the female sexual attractiveness pheromone; smaller females expressed pheromone profiles higher in saturated methyl ketones, while larger females expressed pheromone profiles dominated by unsaturated methyl ketones. Thus, the sexual attractiveness pheromone contains the necessary variation for it to function as a reliable indicator to males of female size. Overall, these results support the hypothesis that the sexual attractiveness pheromone represents the cue used by males to differentiate among potential mates.

Finally, in chapter six I examined whether variation in sexual attractiveness pheromone expression exists between geographically isolated hibernacula and to what extent this variation might result in sexual isolation among the hibernacula. Simultaneous courtship trials revealed that adult males from a hibernaculum in central Manitoba displayed a strong courtship preference for females from their own population over females from the second hibernaculum in western Manitoba, whereas males from

the western Manitoba hibernaculum showed no such preference. Trailing experiments testing the response of males from the two hibernacula to resident and non-resident females showed similar results, demonstrating that the observed male preference is mediated through chemical cues. Subsequent chemical analysis of the sexual attractiveness pheromone showed significant variation in the composition of the pheromone between the two populations, namely in the relative concentration of individual monounsaturated methyl ketones. These results suggest that sexual isolation exists to a degree among denning populations of red-sided garter snakes due to variation in the expression of the sexual attractiveness pheromone.

Overall, the results of this thesis demonstrate that variation exists in the sexual attractiveness pheromone of the red-sided garter snake at multiple levels and strongly suggests that the observed variations have functional significance for the species. The apparent reliance on this pheromone to provide males with answers to multiple questions suggests that this pheromone represents a compound signal containing hierarchical mapping. Hierarchical mapping refers to signals in which differences in mean patterns are used to code for one question and different variants around that mean are then used to answer other questions (Bradbury & Vehrencamp, 1998). Thus, it appears that with a single tongue-flick, a male red-sided garter snake can gain a wealth of information. This includes not only whether the snake under investigation is female, but also the reproductive status of the female, her potential fecundity (based on body size), and her den of residence – all via variation in the expression and detection of a single pheromone, the sexual attractiveness pheromone (Figure 7.1).

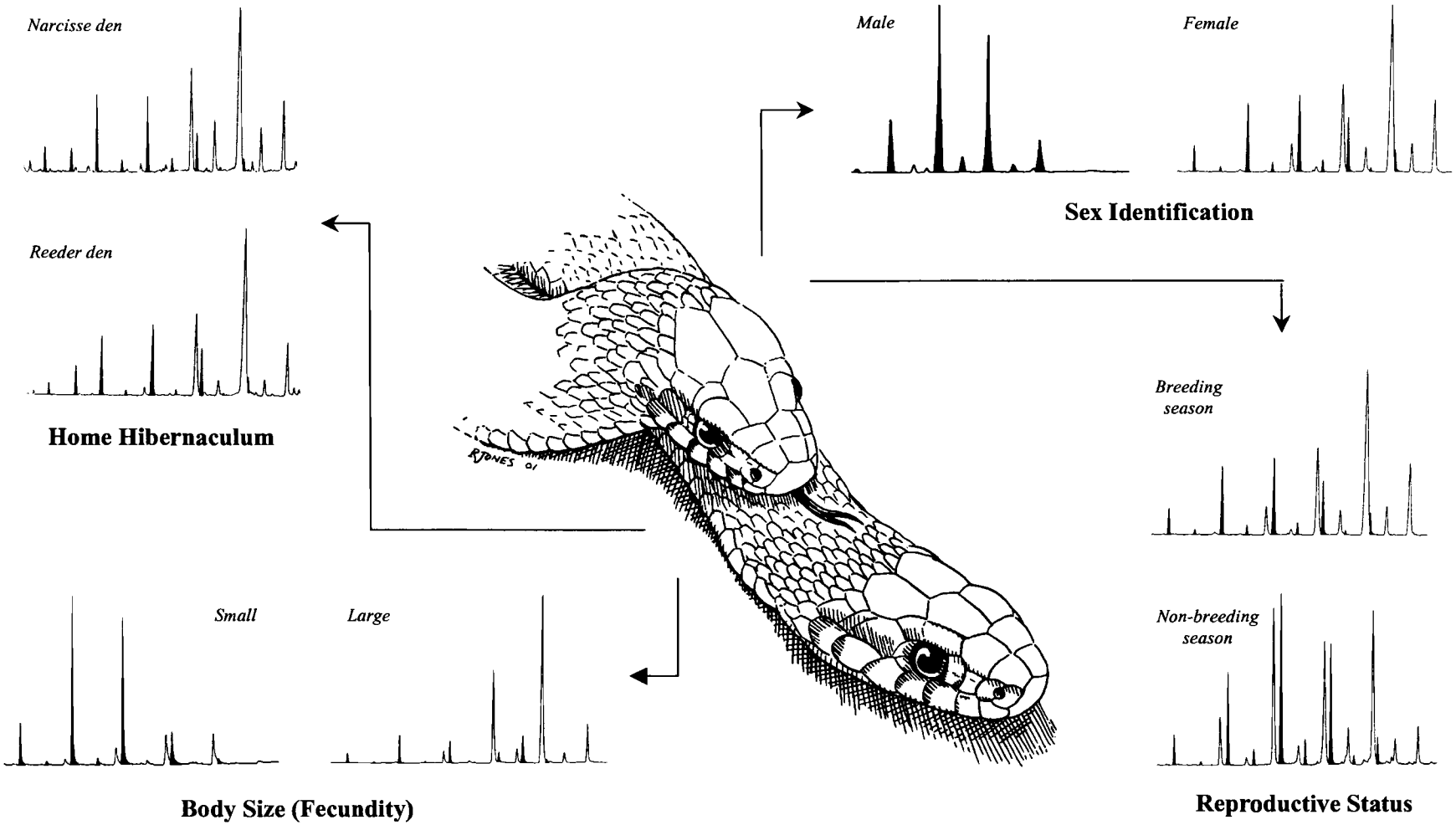

Figure 7.1. Information available to male red-sided garter snakes via variation in the female sexual attractiveness pheromone.

Future Considerations

While the work presented in this thesis indicates that functional significant variation exists in the sexual attractiveness pheromone of the red-sided garter snake, much remains to be explored. For example, while variation was found in the composition of the sexual attractiveness pheromone between the breeding season and non-breeding season, it is unknown to what extent quantitative variation may also be present. Recent work in our laboratory found that during a single breeding season, the relative concentration of methyl ketones contained in the skin lipids of females begins to decrease soon after emergence, suggesting that such a decrease may continue into the non-breeding season. Thus, a male's inability to recognize females, or their trails, during the non-breeding season may result not only from shifts in the composition of the pheromone expressed by a female, but also the total amount of pheromone sequestered in a female's skin lipids.

The proximate mechanisms resulting in the observed variation in pheromone expression among females also need to be examined. The production and expression of the sexual attractiveness pheromone appears to be under hormonal control (estrogen – Crews, 1976, 1985), suggesting that variation in the release rate of hormones and/or sensitivity of target cells to hormones may explain the variation observed between females of varying size or from varying localities. Alternatively, females may differ in various aspects of the biosynthetic pathways involved in methyl ketone production (*e.g.*, expression and/or activity level of enzymes). However, the biosynthetic pathways involved in methyl ketone production have yet to be examined for the red-sided garter snake. German cockroaches (*Blattella germanica*) produce a sex pheromone composed

of similar methyl ketones that are derived from fatty acid biosynthesis (Tillman *et al.*, 1999), which suggests that a similar synthetic process may occur in the red-sided garter snake.

Finally, future research is necessary to elucidate the potential factors leading to intraspecific variation in the production and detection of the sexual attractiveness pheromone. For example, red-sided garter snakes in central Manitoba, unlike individuals in western Manitoba, share hibernacula with a second species of garter snake, the plains garter snake (*Thamnophis radix*). Recent work in our laboratory has revealed that both species have similar pheromone profiles. Thus, intense selective pressure to recognize conspecific females via the sexual attractiveness pheromone in the central Manitoba populations, but not the western Manitoba populations, may drive the pattern of sexual isolation observed among red-sided garter snakes. Alternatively, the asymmetrical sexual isolation observed could result from divergence in this pheromone, and divergence of mating preferences by males for this pheromone, which are both driven directly and indirectly by sexual selection (Arnold *et al.*, 1996).

Regardless of the future direction of research, the results of this thesis, coupled with previous studies, demonstrate that the red-sided garter snake is an excellent model for the study of vertebrate pheromones. The types of studies presented here integrate different levels of biological organization in an attempt to address the questions at hand. Field and laboratory analyses are complementary, bridging the gap between the chemical assessment of pheromones and the ecological issues associated with these molecules. Studies such as these, combining the classical disciplines, are necessary as we continue to expand our understanding of chemical communication in vertebrates.

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